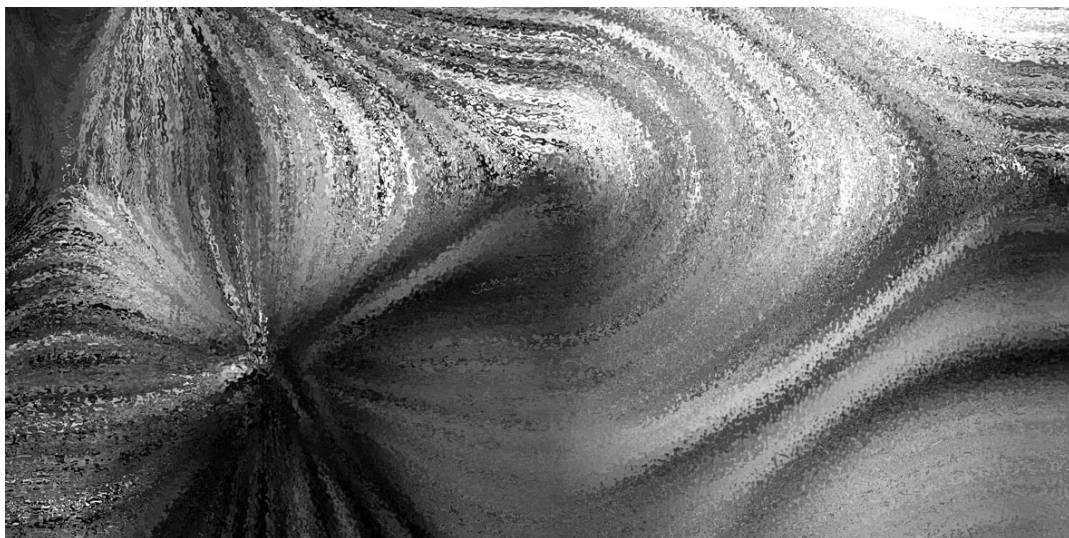


For **AQA** Specifications '**A**'
Advanced Subsidiary

Biology Only



AS Module 2 **Making Use of Biology**

FELTHAM PRESS

Making Use of Biology

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Enzymes in biotechnological processes

Contents

Isolation of enzymes

- ▼ The distinction between intracellular and extracellular enzymes
- ▼ Commercial production of enzymes from microorganisms
 - the growth of large numbers of microorganisms using specific media and septic conditions
 - the isolation and purification of the enzyme product by downstream processing

Application of enzymes in biotechnological processes

- ▼ The applications of enzymes linked to a consideration of their functions
 - enzymes as analytical agents
 - thermostability in industrial processes
 - immobilised enzymes

Introduction

Enzymes are biological catalysts which speed up and co-ordinate chemical reactions in living organisms (see Module 1). They are three dimensional proteins, sometimes with non-protein cofactors attached, whose production is coded for by one or more genes. Several thousands of different enzymes have been identified so far, each catalysing one specific reaction within a narrow range of conditions (e.g. temperature, and pH).

Although enzymes have only been investigated scientifically for just over a century, they have been used by humans for thousands of years in traditional skills, e.g. brewing (the term enzyme means 'in yeast'), and cheese making. While we are still far from identifying and understanding the full range of enzymes and enzyme functions in living cells, knowledge of some enzyme catalysed reactions has led to the development of enzyme-based technology. A range of enzymes now have important commercial applications in industry; including the food and drink industry, the paper industry, and the pharmaceuticals industry. For a range of financial and biological reasons most of the enzymes used in these industries are obtained from fungi and bacteria.

ISOLATION of ENZYMES

Intracellular and extracellular enzymes

Whilst there are many ways of classifying enzymes, one of the most straightforward is a classification based on whether the enzyme functions within or outside the cell which produces it.

Intracellular enzymes are produced inside cells and work inside cells. Examples include DNA polymerase which is involved in DNA replication in the nucleus, all of the enzymes used in cellular respiration, and all of the enzymes involved in photosynthesis.

Extracellular enzymes are produced inside cells but released from cells to work outside of the cell. The most familiar are the digestive enzymes which are produced in cells of the digestive tract or in cells within digestive organs and released into the lumen of the digestive tract. These include salivary amylase produced by cells in the salivary glands of the mouth, and pancreatic lipase and amylase produced by cells of the pancreas and released into the small intestine. In fungi and many bacteria, extracellular enzymes are secreted onto food materials to allow extracellular digestion. Such enzymes are often of great commercial interest.

Enzymes in industry: advantages

Enzymes are biological catalysts, and have a number of unique properties which distinguish them from inorganic catalysts. These properties, which are listed below, make them ideal for commercial use, especially on grounds of economy.

- ▼ Enzymes are highly specific to one substrate. Specificity ensures that only the desired reactions will take place, leading to the production of a pure product. This is particularly crucial in the pharmaceutical industry where enzymes are used to indicate the presence of one substance within a complex mixture.
- ▼ Enzymes work at low temperatures (usually below 40°C) compared to some inorganic catalysts which need temperatures of over 500°C. This reduces the cost of many processes and also makes them safer. In some industrial processes, however, enzymes that work at temperatures over 60°C are used.
- ▼ Enzymes work at atmospheric pressure whilst some inorganic catalysts will only function at much higher pressures. Again this reduces cost.
- ▼ Enzymes are less toxic than many inorganic catalysts, making them more suitable for a variety of processes, particularly in the food and drink industry.

Enzymes in industry: disadvantages

Enzymes do, however, have some disadvantages compared to inorganic catalysts.

- ▼ Enzymes are proteins with a complex 3D shape upon which their activity depends, therefore their efficiency will be affected if the temperature or pH changes from the optimum. At extreme pHs and high temperatures they are irreversibly denatured.
- ▼ Enzyme action may be inhibited by other substances present in a mixture, including the products of their own reactions.

- ▼ The initial stages of research, required to identify suitable and safe enzymes in organisms, are very expensive. In many cases production and isolation of enzymes is also expensive, particularly when they are intracellular enzymes.

Production of enzymes using microorganisms

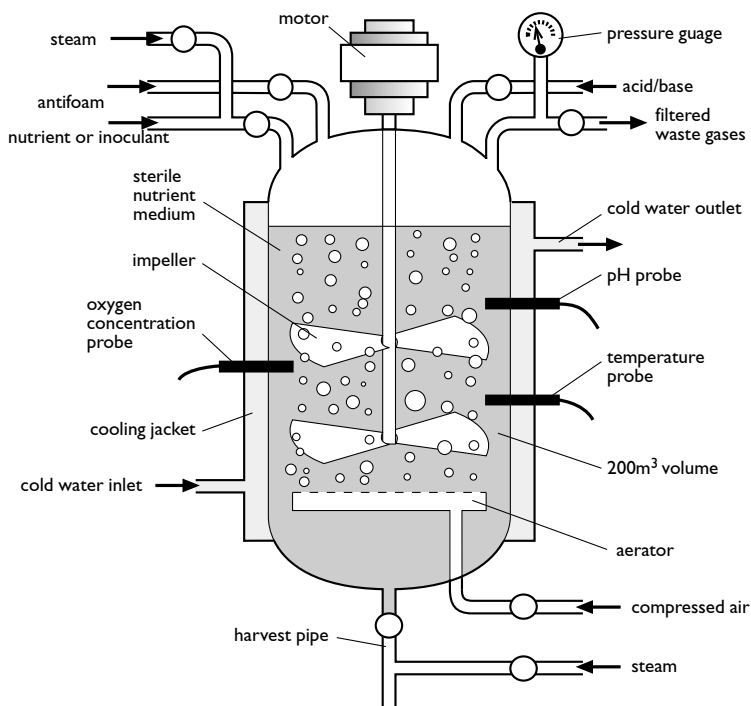
In the past, enzymes required for use in commercial processes were extracted directly from their source in an animal or plant. Hence chymosin or rennilase (formerly rennin) used in cheese making was taken from the stomach of new born calves. Such methods are however very wasteful and expensive and cannot be used to produce enzymes on a large scale. As commercial demand has grown, methods using enzymes produced by microorganisms have developed. In addition to the significant fact that microorganisms naturally produce many extracellular enzymes, other benefits include:

- ▼ If correctly monitored and grown inside specially designed fermenters (see below) such organisms have massive growth rates at low cost.
- ▼ They use only simple nutrient substances or even waste products, contributing to the low costs of the process.
- ▼ They can be easily genetically engineered to produce a range of enzymes originally derived from other species.
- ▼ Mutant strains producing particularly high yields of particular enzymes can often be developed
- ▼ Their exploitation does not raise the same ethical issues associated with the use of animals, e.g. the slaughter of calves to produce chymosin.

Growth of microorganisms

The principles of growing microorganisms in fermenters for the commercial production of enzymes follows the same basic principles as the growth of microorganisms in a laboratory. If optimum growth is to be achieved then the microorganisms require a suitable nutrient medium in which to grow, suitable environmental conditions, and control of competition or predation from other microorganisms.

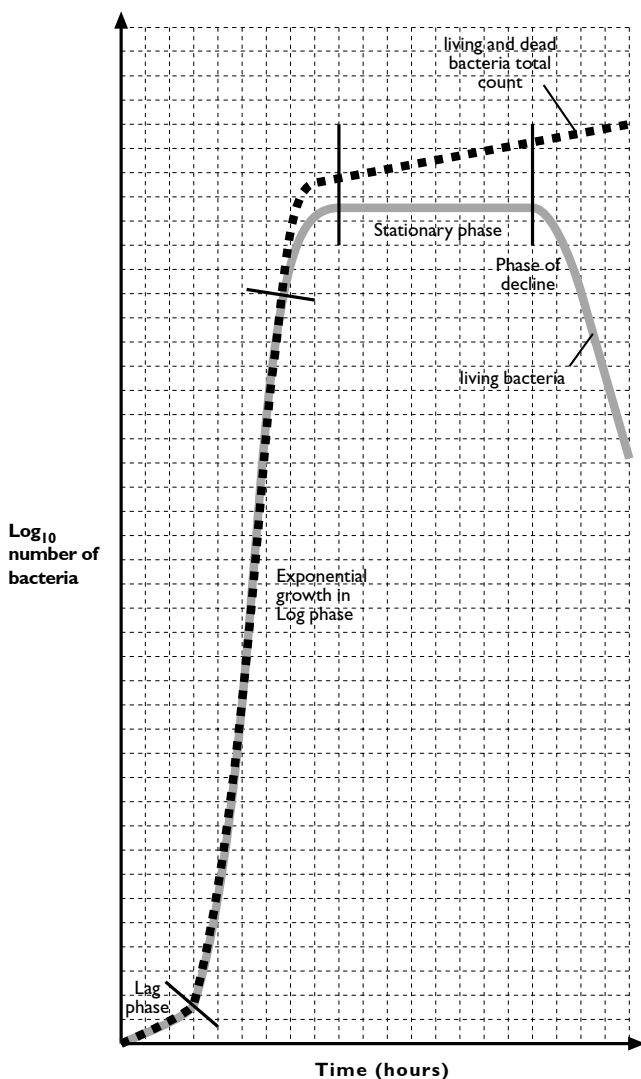
Nutrient requirements vary between species, but all microorganisms require simple organic sources of carbon and nitrogen (such as simple sugars and amino acids), and a range of vitamins and minerals, dissolved in water. Most species will grow best if this medium is kept at around 35-40°C, and at a pH of close to 7. Oxygen requirements will vary from anaerobic conditions to aerobic conditions. It is also important that the fermentation mixture containing the microorganisms and the nutrient medium is well mixed. The term fermentation used to be restricted to anaerobic conditions, but is now used more generally. Most reactions in 'fermenters' are in fact aerobic.

Bioreactor

Given optimal conditions microorganism growth will follow a characteristic pattern. It will begin slowly and then accelerate exponentially. After this point there are two possible scenarios. If the bacteria are left alone, their growth rate will level off and then decline until it crashes to zero, as nutrients and oxygen have been used up, and waste products accumulated. This pattern is used in **batch fermentation** of microorganisms where a single culture of microorganisms and a single addition of nutrient medium is used to produce a batch of enzyme. This is used in almost all cases, producing large batches of commercial enzymes such as chymosin (rennilase) for the cheese industry, and amylase for the baking industry.

In **continuous fermentation** the bacteria are given more nutrients and oxygen, and other conditions are kept optimal, allowing them to continue to grow at a maximum rate. This allows continuous production and extraction of an enzyme. The growth rate of the bacteria can be adjusted by varying the quantity of nutrients added. An example of the use of continuous culture is in the growth of *B. coagulans*, the bacterium which produces glucose isomerase used in the production of high fructose corn syrup.

Growth of microorganisms



◆ CHECKPOINT SUMMARY 30

- ◆ Intracellular enzymes are produced inside cells and work inside cells. Extracellular enzymes are produced inside cells but released from cells to work outside.
- ◆ Growth of large numbers of microorganisms in fermenters for the commercial production of enzymes requires a suitable nutrient medium in which to grow, suitable environmental conditions, and absence of competition from other microorganisms (i.e. sterile conditions).
- ◆ Nutrient requirements vary between species but all microorganisms require simple organic sources of carbon and nitrogen (such as simple sugars and amino acids), and a range of vitamins and minerals dissolved in water.
- ◆ Given optimal conditions microorganism growth will follow a characteristic pattern. It will begin slowly and then grow exponentially.
- ◆ If the bacteria are left alone their growth rate will level off and then decline until it crashes to zero as nutrients and oxygen have been used up.
- ◆ Alternatively, if, the bacteria are given more nutrients and oxygen and other conditions are kept optimal they will continue to grow at a maximum rate.
- ◆ It is essential that the fermenter and all pipes leading to and from it are sterile before microorganisms are introduced. This also applies to all substances added to the fermenter during fermentation. Such sterile conditions are described as aseptic.

Bacteria culture at 30°C

Time/h	Number of cells in millions	
	Living	Living & Dead
0	9	10
1	10	11
2	11	12
5	18	20
10	400	450
12	550	620
15	550	700
20	550	850
30	550	950
35	225	950
45	30	950

Aseptic conditions

It is essential that the fermenter and all pipes leading to and from it are sterile before microorganisms are introduced. This also applies to all substances added to the fermenter during fermentation. Such sterile conditions are described as aseptic. If these conditions are not maintained then microorganisms other than those required will also grow inside the fermenter. Since the conditions are, of course, ideal for growth of microorganisms, unwanted or harmful microorganisms can also rapidly multiply. At worst this could contaminate the product with toxins or poisons that could cause harm to consumers. Less serious but still problematic is the fact that unwanted microorganisms could feed on or compete for resources with the useful microorganisms, so limiting productivity.

To ensure aseptic conditions the fermenter and all inlets and outlets are sterilised with steam under high pressure before use. Air entering the fermenter is filtered, and nutrient media may also be heat sterilised. As the culture organisms could also be a source of contamination they are checked for the presence of other microorganisms. Air leaving the fermenter may also be filtered to prevent microorganisms escaping.

Microorganisms which have high optimum temperatures for growth are particularly favoured in biotechnology as this reduces the chance of contamination by other species. The enzymes so produced will also be thermostable, the advantages of which are discussed below.

Isolation of enzymes

Although the majority of enzymes produced by microorganisms are intracellular, the majority of those used in industrial processes are extracellular. This reflects the fact that isolation and purification of intracellular enzymes is usually difficult and expensive.

Extracellular enzymes are secreted from microorganisms into the culture medium and can usually be isolated from this medium with relative ease. Examples include glucoamylase an extracellular enzyme from *Rhizopus* or *Aspergillus* species, which is used in the processing of corn starch, and chymosin (rennilase) from genetically engineered bacteria, and moulds e.g. *mucor* or yeast, which is used in cheese production.

Intracellular enzymes can only be obtained by damaging the cells in which they are produced. Cells may be disrupted mechanically, or by procedures such as freezing-thawing, osmotic shock, the use of high temperatures, high pHs or digestion by other enzymes. Once the cells have been broken up in this way filtration or centrifugation is used to remove the cell debris, and ultrafiltration or chromatography are used to purify the desired enzyme. Examples of important intracellular enzymes obtained in this way include glucose oxidase (used in biosensors discussed below) and glucose isomerase (used in corn starch processing).

◆ CHECKPOINT SUMMARY

- ◆ The majority of enzymes used in industrial processes are extracellular. This reflects the fact that isolation and purification of intracellular enzymes is usually difficult and expensive.
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- ◆ Once the cells have been broken up in this way filtration or centrifugation is used to remove the cell debris and ultrafiltration or chromatography to purify the desired enzyme.

Application of enzymes in biotechnological processes

Examples of commercial applications of enzymes:

- ▼ Dairy products: forms of rennin and rennin substitutes, e.g. chymosin (rennilase) used to cause milk to coagulate in the production of cheese
- ▼ Fruit juices, wines: pectinases used in the removal of pectins and starches from juice and wines to prevent cloudiness.
- ▼ Brewing: amylases and proteases to digest polysaccharides and proteins in beer and to prevent cloudiness.
- ▼ Sugar: use of amylases and other carbohydrases to produce fructose and glucose from corn (maize) starch. In the processing of cane sugar (sucrose) carbohydrases are also used to remove polysaccharides.
- ▼ Meat: proteases (papain) used to tenderize meat by partially digesting protein fibres before sale.
- ▼ Baking: carbohydrases used to weaken gluten and produce soft textured cakes and breads.
- ▼ Additives: emulsifiers and flavourings for foods may be produced using enzymes.
- ▼ Alcohol production (for drinking and for fuel): amylases and other carbohydrases are used to convert unfermentable starches to fermentable sugars which yeast can utilise. In the production of alcohol for fuel the starting point is agricultural waste, particularly straw.
- ▼ Animal feeds: various enzymes are used to improve the nutritional value of foodstuffs.
- ▼ Paper industry: used in biobleaching, extraction of cellulose from wood (biopulping) and even in de-inking of recycled paper.
- ▼ Leather industry: various proteases used to remove hair from hides and to soften leather.
- ▼ Detergents: proteases, amylases and lipases used in 'biological' washing powders break down food stains on clothes. Also cellulases help to digest loose fibres in cotton, preserving the texture and appearance.
- ▼ Pharmaceutical, analytical and medical: a range of enzymes produced for use in testing kits such as the ELISA test, in biosensors (detecting glucose levels), and test sticks for glucose, proteins and uric acid.
- ▼ Waste treatment: various enzymes used to break down wastes including lignin, cellulose and other solid and toxic materials.
- ▼ Genetics: restriction enzymes (endonucleases) are used to cut DNA for use in genetic engineering, genetic fingerprinting. Polymerases are used to amplify DNA (section 11.3).

Enzymes as analytical reagents

Enzymes are ideal tools for use in biochemical analysis in both industry and medicine. They have indeed revolutionised many clinical tests which aim to detect the presence of certain compounds in biological fluids (blood, plasma, urine etc) and are widely used in hospitals and laboratories. They are also used in industrial processes, including the production of ethanol and other materials in fermenters. The two main advantages of enzymes over other analytical reagents are:

- ▼ Enzymes have a high specificity: They will only react with one specific substrate, even if that substrate is found in a complex mixture.
- ▼ Enzymes have a high sensitivity: they can catalyse reactions even when the substrate is present in very low concentrations.

Enzymes are used in three main types of system:

Diagnostic test strips: basic plastic strips containing a pad with an immobilised enzyme(s) (see below for discussion of immobilised enzymes), show a colour change if the substrate is present, and give a semi-quantitative result via a graded colour change. These are very cheap but can only be used once.

Enzyme electrodes or '**biosensors**': contain an immobilised enzyme, a transducer and a tiny electric circuit. The enzyme catalysed reaction produces a product which generates an electric current in the transducer. The current is proportional to the amount of product (and hence substrate) present and is converted into a digital read out. These are small and reusable tools.

Enzyme analytical reactors: Large scale laboratory tools using soluble enzymes and colour changes to test hundreds of samples per day.

Examples of enzymes in enzyme electrodes used in medicine and industry:

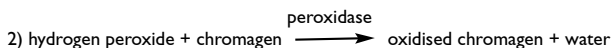
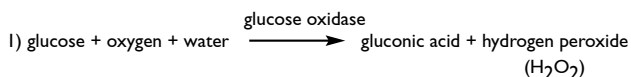
Enzyme(s)	substrate/anylate
Alcohol dehydrogenase:	alcohol
Cholesterol esterase and cholesterol oxidase	cholesterol
Glucose oxidase and peroxidase	glucose
Urease	urea

Using enzymes to test for glucose

One of the most common uses of enzymes as analytical reagents in hospitals is in tests for blood glucose. The level of glucose in the blood is closely regulated in a homeostatic fashion by the pancreatic hormones insulin and glucagon. The normal level is around 90-150mg/100cm³ of blood. Both high and low levels of glucose are harmful and are useful indicators of disease. In particular very high levels of glucose in the blood following meals are characteristic of the disease diabetes. This disease is also characterised by the presence of glucose in the urine. Glucose levels in blood and urine can be detected using the two enzymes: glucose oxidase and peroxidase.

▼ **Diagnostic test strip: for testing glucose levels in urine**

The colourless pad on the test strip contains the enzymes glucose oxidase and peroxidase immobilised in a cellulose mat along with a colour reagent (e.g. chromagen). When the pad is dipped into a substance containing glucose the reactions shown below occur. The end point is a blue colour (resulting from the reaction of the hydrogen peroxide with chromagen). The shade of blue reflects the concentration of glucose present and is thus semi-quantitative. The pad will only take about 15 seconds to change colour.



Test strips of this kind are used regularly by diabetics to check their urine for the presence of glucose. A positive result would indicate that their blood glucose level was too high. Other simple test strips are available to test blood or urine for a range of compounds including urea (using urease enzyme), and cholesterol (using cholesterol esterase and cholesterol oxidase).

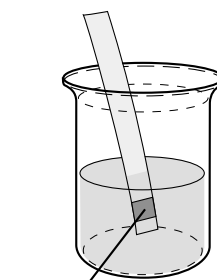
▼ **Glucose testing using a biosensor**

The biosensor uses the immobilised enzyme glucose oxidase. If a substance containing glucose is placed on the test area of the biosensor the reaction noted above takes place. The gluconic acid produced by the reaction generates an electric current in the transducer which is translated into a digital read-out. The read-out takes less than 20 seconds to appear and gives a quantitative measure of the amount of glucose present in the sample. This method requires a very small sample, a drop of blood from a pin prick is adequate.

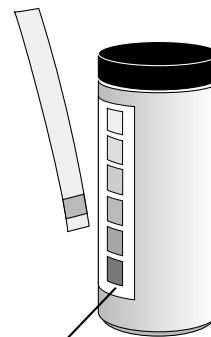
Biosensors like this, which are easy to use, relatively cheap yet also highly sensitive and specific, are now commonly used in hospitals to measure the levels of a range of biologically important substances in body fluids.

Immobilisation of enzymes

Since enzymes, like all catalysts, are not used up in the processes they catalyse they can, potentially be reused again and again. This is particularly desirable since enzymes are expensive to produce. One of the difficulties of reusing enzymes is, however, the difficulty of recovering the enzyme from the substrate/product where it is in a very low concentration. This can be a time consuming and expensive exercise. If the enzyme is not recovered then not only is the process wasteful, but it may also be harmful as the final product will be contaminated with enzyme.

Testing for glucose using a diagnostic strip

enzyme and chromagen impregnated pad



analysis made by comparison with colour chart on container

For these reasons the development of immobilised enzymes has improved enzyme technology enormously. Immobilised enzymes are enzymes that have been attached to another (insoluble) material in some way and hence prevented from moving freely within the substrate mixture. They may be physically immobilised within a system by being attached to a fixed membrane or gel made of a range of substances including silica, starch, and cellulose. Processes used to attach the enzymes are adsorption, covalent bonding, and entrapment. In other cases the enzyme may move within the substrate but only once encapsulated within beads of another material. In the 'attached' systems enzymes never become mixed with the product, whereas encapsulated enzymes are mixed in with the final product but are easily extracted by filtering or other methods.

There are many advantages to immobilising enzymes, in addition to cost and ease of extracting a pure product.

- ▼ Immobilised enzymes may be more easily added to and removed from reactors to allow the rate of reaction to be more carefully controlled. This means that enzymes can be used to partially, but not completely, digest a substrate.
- ▼ Immobilised enzymes can lead to much greater production efficiency. Primarily they can be used in a continuous enzyme reactor where substrate is constantly added, and product constantly produced, without the need to add or extract enzyme. The best example of this is in the production of high fructose corn syrup described below.
- ▼ Immobilised enzymes tend to be more stable. In particular they are less likely to be inactivated or denatured by high temperatures (they are more thermostable), e.g. glucose isomerase discussed below is stable at 65°C when immobilised but is denatured at 45°C when free; by changes in pH, or the presence of other chemicals.
- ▼ Immobilised enzymes can also be used for longer before their activity decreases, and are less likely to lose activity during periods of storage.

A disadvantage of immobilising enzymes is that the rate of reaction may be slower because the enzyme has reduced kinetic energy and is often partitioned from the substrate by a membrane through which the substrate must diffuse.

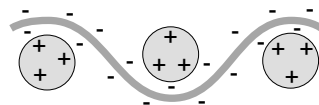
The importance of thermostability

The continued profitability of the 'high fructose corn syrup' (HFCS) industry and other industries relying on enzyme based reactions has depended on the discovery of increasingly thermostable (heat tolerant) enzymes which can be used at temperatures up to and above 60 °C. Many of these enzymes come from bacteria which inhabit hot springs or live deep in the earth's core, where enzymes tolerant of temperatures up to 250°C have been discovered. Enzyme thermostability is an essential feature of such industries for the following reasons:

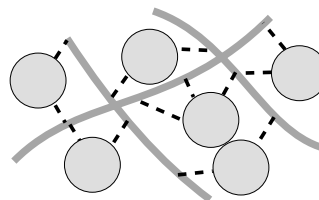
- ▼ The higher the temperature, the lower the chance of contamination with micro-organisms which are generally inhibited at 60°C.
- ▼ The higher the temperature the faster the rate of reaction. This makes the plant more efficient, as more substrate can be processed in a given time.

Immobilised enzymes-different systems

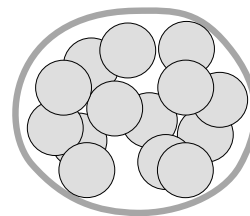
Absorption



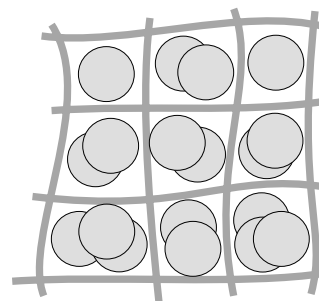
Covalent bonding



Encapsulation



Entrapment



- ▼ If the enzyme is thermostable then it may allow the same enzyme to be used for more batches before its activity declines.
- ▼ The higher the temperature the less viscous the substrate. This is an important factor in the production of HFCS. A very viscous substrate is difficult to stir and thus it will take longer for enzymes to come into contact with the substrate. This is important in the first two stages of HFCS production. The higher the temperature the higher the solubility of reactants. Again this speeds up the reaction, improving productivity.

Corn starch processing: the importance of thermostable and immobilised enzymes

The digestion of corn (maize) starch to produce high fructose corn syrup (HFCS) is one of the largest scale uses of enzymes in industry. In the USA several HFCS plants each produce over 1 million tonnes of the syrup per year. The process is totally reliant on enzymes, and involves three main stages:

- ▼ **Thinning** of the viscous corn starch using amylase enzyme. This stage is carried out at a temperature of 105°C using enzymes from *Bacillus stearothermus* or *B. licheniformis*. The high temperature is particularly valuable in decreasing the viscosity of the mixture.
- ▼ **Hydrolysis** of the pre-thinned starch (dextrins) to a glucose syrup using glucoamylase, an extracellular enzyme from *Rhizopus* or *Aspergillus* species. This enzyme is not immobilised but used as a soluble enzyme in batches at temperatures of 55-60°C. The main reason for this is that the reaction is faster when the enzyme is not immobilised. Also as the enzyme is very cheap, the need for new batches of enzyme each time is not a major expense. The use of batches also allows production to be monitored more easily. Afterwards the product is filtered and passed through an ion exchange mechanism to remove the enzyme.
- ▼ **Conversion or isomerisation** of glucose syrup to fructose syrup (HFCS). Fructose is much sweeter than sucrose or glucose so it is a more valuable product. This stage, leading to the production of syrup containing about 55% fructose is performed by the immobilised enzyme glucose isomerase. This enzyme is attached in various ways to the interior of large columns through which the glucose syrup is continually passed. Fructose syrup (HFCS) continuously emerges from the column in a relatively pure form as it is not contaminated with enzyme. This occurs at 60°C as the enzyme used is not very thermostable.

'Biological' detergents

Enzymes which are stable at higher temperatures are also valuable in the production of biological washing powders. Such powders use proteases, lipases, amylases and cellulases to aid the removal of food and other stains from clothes. Although some early forms used enzymes that could only resist temperatures of 40°C, newer products can be used in washes at 60°C or above with improved results.

◆ CHECKPOINT SUMMARY

- ◆ Enzymes are ideal tools for use in biochemical analysis in both industry and medicine. They have a high specificity and will only react with one specific substrate, even if that substrate is found in a complex mixture.
- ◆ Enzymes have a high sensitivity: they can catalyse reactions even when the substrate is present in very low concentrations.
- ◆ Enzymes are used in three main types of system: diagnostic test strips: enzyme electrodes or 'biosensors': enzyme analytical reactors.
- ◆ Industrial processes require a high degree of thermostability (heat tolerance).
- ◆ The higher the temperature the lower the chance of contamination with micro-organisms, the faster the rate of reaction, the less viscous the substrate and the higher the solubility of reactants.
- ◆ Immobilised enzymes are enzymes that have been attached to another (insoluble) material in some way and hence prevented from moving freely within the substrate mixture. They may be physically immobilised within a system by being attached to a fixed membrane or gel made of a range of substances including silica, starch, and cellulose.
- ◆ Immobilised enzymes may be more easily added to and removed from reactors to allow the rate of reaction to be more carefully controlled.
- ◆ Immobilised enzymes can lead to much greater production efficiency. Primarily they can be used in a continuous enzyme reactor where substrate is constantly added and product constantly produced without the need to add or extract enzyme.
- ◆ Immobilised enzymes tend to be more stable (better able to resist alteration to shape and activity.) In particular they are less likely to be inactivated or denatured by high temperatures (they are more thermostable), changes in pH, or presence of other chemicals.
- ◆ Immobilised enzymes can also be used for longer before their activity decreases and are less likely to lose activity during periods of storage.
- ◆ A disadvantage of immobilising enzymes is that the rate of reaction may be slower because the enzyme has reduced kinetic energy and is often partitioned from the substrate by a membrane through which the substrate must diffuse.

DNA control of enzyme synthesis

Since the base sequence of DNA in a gene controls the production of one specific polypeptide or protein, it follows that any change in this base sequence (a gene mutation) may lead to the production of a faulty protein. Even a substitution or deletion of a single base can alter the translation of the DNA code into a sequence of amino acids in a polypeptide chain. If the primary structure of a polypeptide is altered this can affect its secondary, tertiary and quaternary structures, with corresponding effects on its function. The presence of such a faulty protein may in turn affect the appearance and functioning (phenotype) of the organism as a whole and cause a genetic disease or disorder. Gene mutations which occur during the production of gametes are thus the cause of genetic diseases in the offspring.

Some genetic diseases are referred to as 'inborn errors of metabolism' because they affect one or more of the metabolic pathways involved in the normal functioning of the organism. They may, for example, include diseases where particular enzymes necessary for metabolic functions are faulty or absent, e.g. Phenylketonuria (PKU). In this example the pathway from faulty gene to abnormal phenotype is clear. Identification of the gene and protein product responsible for the disease can allow medical intervention at any point in the pathway from gene to disease.

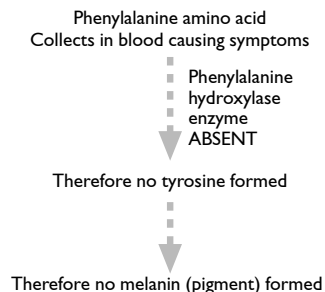
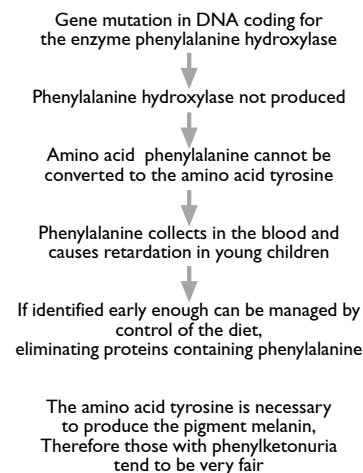
Phenylketonuria (PKU)

Most humans are able to convert some types of amino acids into other types of amino acid in the liver and thus produce all the proteins necessary for growth, repair and functioning of tissues. This explains why some amino acids are said to be essential (must be taken in the diet) whilst others are non-essential (do not need to be taken in the diet as they can be made from the essential amino acids in the body). Conversion of one amino acid to another (transamination) allows the body to make full use of ingested proteins and also prevents the build up of particular unused amino acids which could be dangerous.

In the disease phenylketonuria (PKU), however, a gene mutation in the DNA coding for the enzyme phenylalanine hydroxylase means that this enzyme is not produced in the liver. Without phenylalanine hydroxylase the body cannot convert the amino acid **phenylalanine** to the amino acid **tyrosine**. This results in an accumulation of phenylalanine in the body which severely affects many systems. Untreated sufferers may be both mentally and physically retarded.

PKU can be treated by modifying the diet to ensure that it lacks, or is very low in, the amino acid phenylalanine. The amino acid tyrosine may also be given. Under these circumstances individuals will not develop any signs of retardation. In the UK, all new-born babies are screened for PKU using a simple blood test which allows individuals with this genetic condition to be detected and treated before damage occurs. Treatment is only necessary in children, as adults seem to be unaffected by the high phenylalanine levels.

PKU sequence and effects



◆ SUMMARY OF PKU

- ◆ Gene mutation occurs in DNA coding for the enzyme phenylalanine hydroxylase
- ◆ Enzyme phenylalanine hydroxylase not produced
- ◆ Faulty metabolic pathway: amino acid phenylalanine not converted to tyrosine
- ◆ Phenylalanine accumulates with toxic effects
- ◆ Abnormal phenotype: Mental and physical retardation occurs.

Gene technology

Content:

Recombinant DNA

- ▼ The production of recombinant DNA and its use in the production of human insulin and other proteins
 - isolation of the required gene
 - use of the enzymes: reverse transcriptase, restriction endonuclease and ligase
 - sticky ends: insertion of the gene into a vector and its subsequent introduction into host cells; plasmids and viruses as examples of vectors
 - use of genetic markers such as genes conferring antibiotic resistance to detect genetically modified organisms
 - multiplication of host cells.
- ▼ The moral and ethical issues associated with recombinant DNA technology.

RECOMBINANT DNA

Imagine how simple life would be if there were only one type of computer and only one type of software. All the machines could then use the same language and all the information would be interchangeable. This is exactly how it is with the language of the genes. The synthesis of polypeptides from amino acids relies on exactly the same mRNA codons in every living organism so, for example, 'GCG' codes for the amino acid alanine and 'CGA' codes for arginine whether it occurs in a human, a honey bee or a mushroom. Now that genes can be identified and isolated, gene technology is providing a means for transferring them from one organism to another.

The terms 'genetic modification', 'genetic engineering' and 'gene technology' refer to the processes and techniques by which genes may be extracted from the DNA of one organism and inserted into the DNA of another organism (the **host** organism). In this way, the gene set (**genome**) of the host can be **transformed** (genetically modified). The modified DNA is called **recombinant DNA** because it has been re-combined to make a different set of codes.

Examples of genetically engineered products

Insulin is needed by people who are unable to produce sufficient quantities of the hormone and who would otherwise develop the disease **diabetes mellitus**. This is a condition, affecting 2% of the population, in which blood sugar rises to dangerous levels. Before gene technology, the only source of insulin was natural. It was extracted from the pancreases of pigs and cattle. This was not ideal because human insulin is slightly different in chemical structure, and although the pig and cattle version works on glucose in a similar way, some people are allergic to it and the process is both costly and open to contamination by impurities.

In the early 1980s the gene for human insulin was transferred from human pancreas cells to *E. coli* bacteria. Bacterial colonies modified in this way were cultured for mass production in industrial fermenters providing a constant supply of insulin. Now most insulin is produced by transformed bakers' yeast cultures. Yeast has various advantages over bacteria. It does not produce any toxic by-products, it can be grown on simple nutrient media, and it can secrete the finished product directly into the culture medium making the harvesting process easier.

Factor VIII is a vital blood clotting factor lacking in people suffering from **haemophilia**. Natural sources are both expensive and liable to contamination but now the gene for factor VIII has been engineered into yeast cells.

The food industry uses more and more genetically engineered products. One of the first to be developed on a commercial scale was the enzyme **chymosin** (rennilase) which is used to coagulate milk for the production of cheese. Chymosin occurs naturally in the stomach of young mammals, where it is often referred to as rennin. The cheese making industry relied on extracts from calves' stomachs until the advent of gene technology. Now chymosin can be obtained from modified yeast cultures grown in fermenters.

The techniques of Genetic Manipulation

Gene technology relies on the fact that the genetic language is universal; the 'software' (genes) can be used in any living organism. The difficulty lies in the 'cutting and pasting' of genetic information from one organism to another, and you must first become familiar with three enzymes which are crucial to this process.

Reverse transcriptase

Reverse transcriptase is an enzyme which makes a single strand of DNA from an RNA template. From this strand of DNA, a complementary strand can be assembled with the aid of DNA polymerase. Reverse transcriptase enzyme was identified and isolated from **retroviruses**, a group of viruses which includes the HIV virus. The genetic material of retroviruses is RNA (not DNA), and when retroviruses invade cells their RNA uses reverse transcriptase to make a piece of DNA. This becomes incorporated into the host cell's own DNA and directs the production of new viruses (an example of natural genetic modification of the host cell).

Reverse transcriptase is used in gene technology to make DNA from mRNA. Remember that mRNA is a copy of a single gene and that mRNA of a working gene may be found in the cytoplasm of a cell. It is possible to isolate a gene in the form of mRNA and then to multiply it using a combination of reverse transcriptase and DNA polymerase.

Restriction endonuclease enzymes

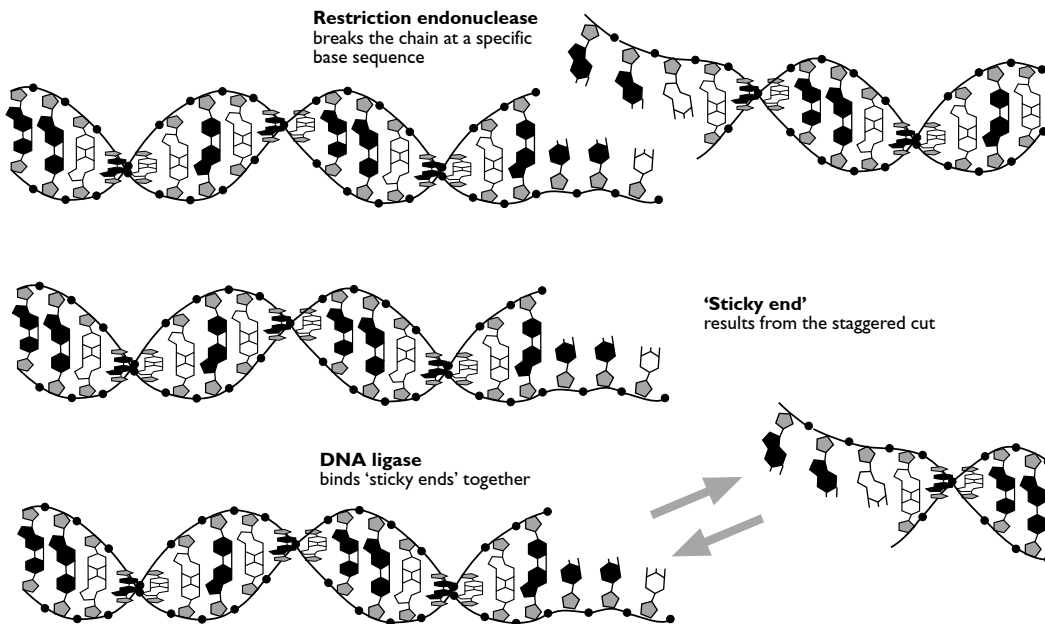
Another vital tool for gene technologists are enzymes called restriction endonucleases (restriction enzymes) which act like scissors to chop up DNA at specific base code sites into smaller polynucleotide fragments. Restriction enzymes are extracted from bacteria. In bacteria they serve to protect the organism from invading viral DNA. In gene technology they are used to cut DNA into fragments which can be separated and isolated. Often the cut leaves '**sticky ends**' which are exploited in another stage of the process.

DNA ligase

To 'ligate' is to join. DNA ligase is a joining enzyme. It is used to stick the sticky ends of DNA fragments together creating new combinations (**recombinant DNA**).

The techniques for transferring genes from one organism to another can be considered in five main stages.

Action of restriction and ligase enzymes



Isolation of the required gene

It is surprisingly easy to extract DNA from living cells as you may already have discovered in the laboratory. Using restriction enzymes and gel electrophoresis it is possible to separate and isolate different DNA fragments, even pure genes. Alternatively, genes can be extracted from cells in the form of mRNA as described above and converted into DNA using reverse transcriptase.

The use of a vector

A vector is a carrier. The most common vectors in gene technology are bacterial plasmids and

bacteriophage viruses, both of which can carry the required gene to the host cell.

Plasmids are rings of DNA found in bacterial cells which are not part of the main bacterial chromosome. They are easily transferred from one bacterial cell to another. Plasmids can be cut at specific sites using restriction enzymes to leave sticky ends. If the same enzyme is used on the isolated gene then the sticky ends will match and the two can be joined using DNA ligase, so that the plasmid contains the recombinant DNA.

Bacteriophages are viruses which infect bacteria. They have a simple ring of DNA which they inject into the bacterial cell. This ring of DNA resembles a plasmid and can be modified in the same way to contain the required gene.

Transformation of the host cells

The vector is now brought into contact with the host cells, in the hope that some of the recombinant DNA might be taken up by the host cells. In order to discover whether or not the host cells have been transformed, **marker genes** (selection genes) are used. These are genes which are engineered into the vector alongside the required gene. They do not harm the host cell in any way but code for products which show up in an easily identifiable form in the host cell, confirming that the process of transformation is complete. One of the more common marker genes codes for antibiotic resistance. The transformed host cells can be isolated by introducing an antibiotic to the culture. All non-transformed cells are killed by the antibiotic, but the transformed cells survive as a result of the antibiotic resistant marker gene.

There has been some concern expressed about the use of antibiotic resistant genes. Opponents of this technique point to the risk of such genes escaping and entering disease organisms. Imagine the potential of a disease-causing bacterium, genetically modified to resist antibiotic treatment.

Multiplication of transformed cells

Once a cell or group of cells has taken up recombinant DNA, the next step is to culture the cells in a suitable growth medium, allowing them to grow and multiply. In the case of transformed bacterial and yeast cells, this can be done on a large scale in industrial fermenters, allowing the product(s) of the new gene to be harvested and purified, as in the case of insulin, cofactor VIII and chymosin.

Questions raised by Gene technology

Gene technology raises many hopes for the future. It should be possible for example to expand the tolerance range and extend the habitat of many crop plants by engineering resistance to salinity, heat, cold, and wind. New varieties will compete better with weeds and be more tolerant of attack from insects and fungal disease. Nitrogen fixation will be enhanced, and renewable energy crops will begin to make a realistic difference to the greenhouse gas levels. Malnutrition may be overcome by staple food crops with a complete complement of essential amino acids, and new drugs will be developed from plant substances.

It also raises a number of questions about the way we want to live. Genetic modification has brought in the age of the designer plant, potatoes exclusively designed for oven chips, square tomatoes, straight bananas, salad vegetables, and fruits of every colour and texture. If there is a market for it, then the plant scientists will be able to engineer it. However, the accidental transfer of new genes into wild organisms could lead to herbicide resistant weeds and antibiotic resistant bacteria. Transgenic plants can also lead to transgenic pests and microbes.

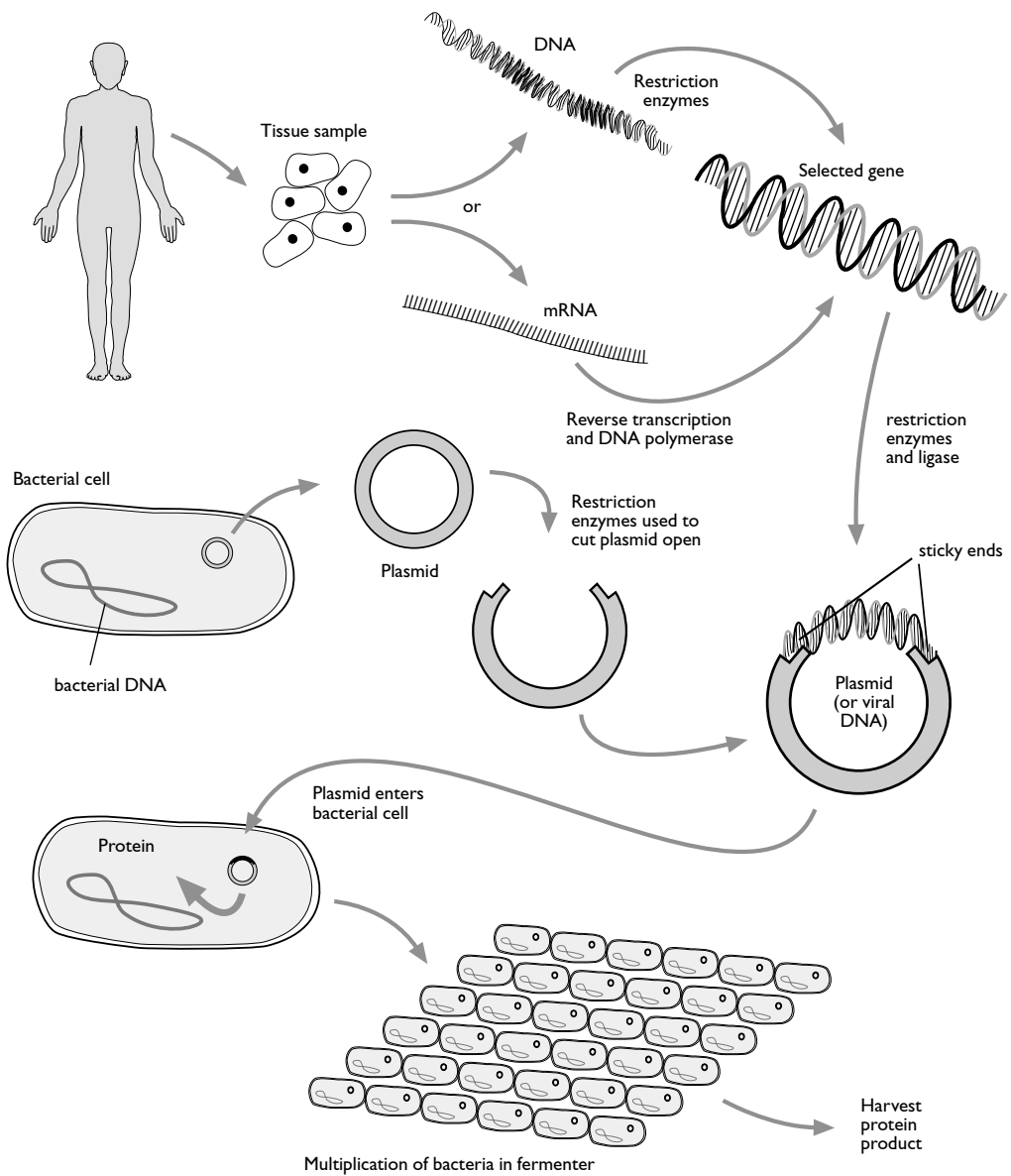
The world population, currently about 5.5 billion, is expected to double to 11 billion by the year 2050. Given that 1 billion are already classified by international agencies as 'poor' and 500 million live in 'absolute poverty', it is clear that food production must be more than doubled in the next 50 years, and this must be achieved on a reducing area of fertile land.

Plant biotechnology, already moving at a rapid pace, offers some hope for the future. Plant biotechnology however, is largely the property of 'First World' independent companies, and 97% of the world's population increase will occur in the developing nations. Furthermore, environmental safety procedures, which evolve with the industry, exist in, and apply only to the countries which have the technology, yet transgenic products now have world wide distribution. A great deal of international cooperation, not to mention finance, will be needed to overcome the problem of safely transferring the products of the new technology to the countries which need them most. The human genome project which is analysing the sequence of bases in human DNA, also raises many problems to be dealt with. Certainly some inherited conditions and tendencies will be better understood, and various treatments developed. On the other hand the potential for abuse is enormous, including refusal of insurance cover to certain groups, employment vetting, immigration control and many others.

◆ CHECKPOINT SUMMARY

- ◆ Isolation of the gene to be manipulated is the first step
- ◆ It is easier to find and isolate the appropriate mRNA which has been transcribed (copied) from the relevant DNA, than it is to try and isolate the particular length of DNA itself
- ◆ The enzyme reverse transcriptase can then be used to make a copy of the DNA from the mRNA
- ◆ Plasmids (circular DNA) are isolated from bacteria
- ◆ Plasmids are cut open by restriction enzymes and the length of DNA is inserted and the plasmid resealed by ligase enzymes
- ◆ The genetically modified plasmids are then reintroduced into the bacteria
- ◆ The bacteria multiply and produce the protein coded for by the inserted DNA
- ◆ To identify those bacteria which have successfully taken up the modified plasmids marker genes are incorporated along with the required gene
- ◆ Marker genes include those for antibiotic resistance so that the culture can be flooded with antibiotic and only the bacteria with the marker gene will survive
- ◆ Two early successes with these techniques were the synthesis of human insulin for the treatment of diabetics, and blood clotting factor VIII for the treatment of haemophiliacs.

Genetic Engineering



Forensic Examination of Blood

Contents

Principles of immunology

- ▼ Definition of antigen and antibody. The immunological response of B-lymphocytes
- ▼ ABO blood group antigens and agglutination

Genetic fingerprinting

- ▼ Genetic fingerprinting
 - restriction enzymes
 - electrophoresis
 - radioactive DNA probes

Polymerase chain reaction

- ▼ The polymerase chain reaction and its importance in obtaining increased amounts of DNA for analysis

PRINCIPLES OF IMMUNOLOGY

Definition of antigen and antibody

In order to remain healthy and able to function properly, the human body must protect itself from foreign material, particularly disease-causing organisms, known as **pathogens**. Initially it must aim to prevent pathogens from entering the body in the first place, and then it must be able to defend itself against them if they do enter. The first lines of defence are therefore the barriers to infection.

If pathogens do manage to pass through these barriers and enter the body tissues including the blood, an immune system is necessary to attack and destroy these pathogens and so prevent serious disease or death. There are two branches of the immune system in operation within the body which co-operate with each other to some extent.

◆ SUMMARY OF BARRIERS TO INFECTION

- ▼ impermeable skin which covers the body, unless broken, when the blood clotting process is rapidly activated to plug the wound and prevent pathogen entry
- ▼ the mucous membranes of nose and respiratory tract trap pathogens in sticky mucus
- ▼ anti-bacterial enzymes in saliva, sweat and tears
- ▼ stomach acid (pH 2).

These are:

- ▼ **Non-specific immunity** which is not targeted at specific types of pathogen but at foreign material in general. This involves phagocytosis and inflammation and is immediate.
- ▼ **Specific immunity** which uses B and T lymphocytes to target specific pathogens. Such responses are more complex and are often delayed. B-lymphocytes are concerned with **humoral immunity**, involving antibodies, whilst T- lymphocytes are concerned **cell-mediated immunity** with the direct killing of infected cells.

Immunology is the study of the mechanisms by which the B and T lymphocytes recognise and and destroy foreign material which enters the body. In all cases, foreign or 'non-self' material (in the form of pathogens, in the form of human cells from other individuals (as in blood transfusions or organ transplants, or, in the case of allergic reactions, in the form of molecules on common products like peanuts) is distinguished from 'self' material by the presence of 'non-self' antigens.

Antigens

Antigens are defined as molecules which, when foreign to an individual, stimulate an immune response by lymphocytes in the body. They are often protein or glycoprotein molecules found on the cell surface membrane of 'non-self' cells, but a range of other molecules also act as antigens. Antigens on the cell surface are important in cell recognition. Under normal circumstances the immune system of an individual will recognise all of their own cells as being 'self' owing to the familiar types of antigens present. Any cells (or other materials) with different 'non-self' antigens are rapidly detected by white blood cells known as B-lymphocytes and T-lymphocytes and targeted for destruction.

The immunological response of B lymphocytes

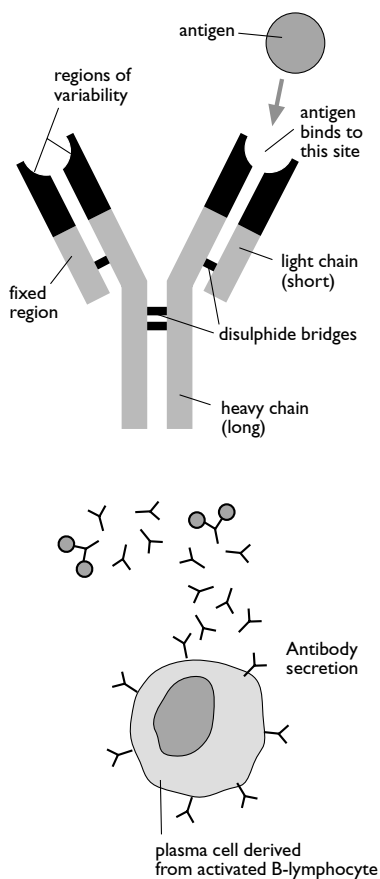
B-lymphocytes are involved in the production of antibodies in response to foreign antigens which is known as humoral immunity.

On the cell surface membrane of B-lymphocytes are a number of specific antigen receptors or membrane bound antibodies. These are sites which may become attached to antigens on the surface of pathogens or other foreign material, leading to a sequence of events in which free antibodies are produced to destroy the foreign material.

There are many thousands of specific types of B-lymphocyte and each is capable of recognising only one specific antigen. This allows specific antibodies to be produced in response to a vast range of antigens. For example, there is one type of B-lymphocyte which will attach to the bacterium causing TB and cause the release of antibodies against TB, and another type which attach to a red blood cell of blood group B in the body of a person with blood group A, and cause the release of antibodies against the group B antigens. This specificity is due to slight differences in the shape of the antigen receptor protein on each type of B-lymphocyte.

Antibodies are a type of protein molecule known as **immunoglobulins** which are secreted by activated B-lymphocytes when a specific antigen is encountered. They are shaped like a Y with the straight tail of the Y being known as the **constant region** since it is the same in every type of antibody. The forked part of the Y is known as the **variable region** contains antigen-binding sites which differ slightly in shape between each type of antibody. Antibodies do not directly destroy foreign material but target it for destruction by other branches of the immune system.

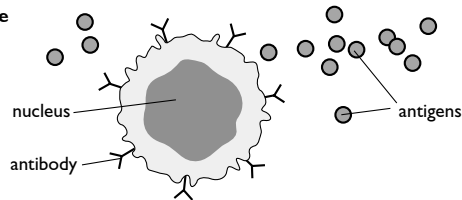
Structure of an antibody



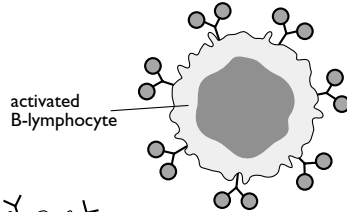
◆ SUMMARY OF THE IMMUNOLOGICAL RESPONSE OF B-LYMPHOCYTES

- ▼ Foreign material bearing antigens enters body
- ▼ Antigens on surface of foreign material come into contact with the specific antigen receptors on a B-lymphocyte
- ▼ Binding of antigen to antigen receptor activates the B-lymphocyte and causes it to divide by mitosis producing a clone of identical B-lymphocytes
- ▼ Most of the B-lymphocytes turn into plasma cells and the rest turn into memory cells
- ▼ The plasma cells begin to secrete specific antibodies against the foreign material concerned. Antibody molecules are secreted at a very high rate: up to 30,000 molecules per second
- ▼ The antibodies attach to the antigens on the foreign material and lead to the destruction of it in one of three ways:
 - By coating foreign cells or objects and causing them to stick together in such a way that they can then be engulfed by phagocytes (another type of white cell)
 - By binding to the foreign cells or objects at sites which interfere with their activities. For example, by covering the protein coat of a virus and preventing it from attaching to host cells.
 - By stimulating the release of other blood components called complement to lyse and destroy foreign cells
- ▼ Once the foreign material has been destroyed, the plasma cells eventually die and antibodies stop being secreted. But the memory cells remain in the lymph nodes and the circulation. If a subsequent infection with the same pathogen occurs then some of these will turn into plasma cells and start producing antibodies. This is the basis of natural active immunity to diseases and underlies the practice of vaccination (artificial active immunity).

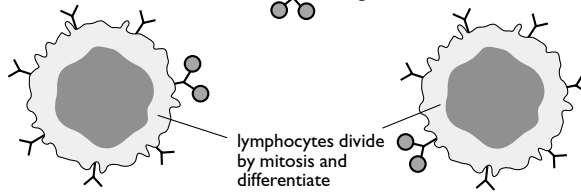
Immature B-lymphocyte



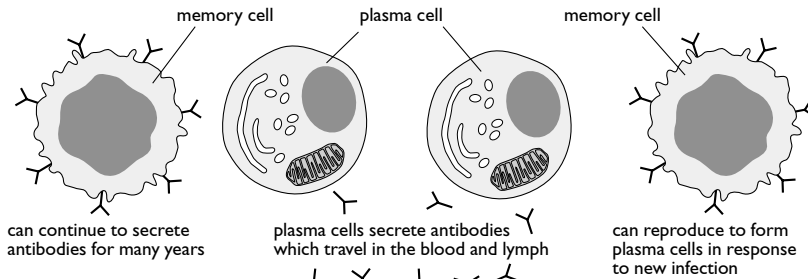
Activation



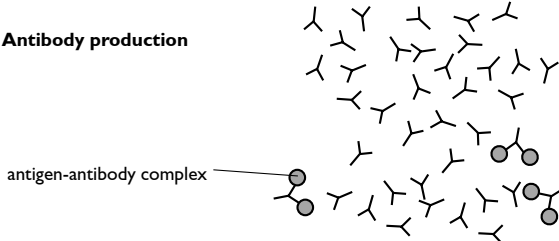
Division



Differentiation into plasma cells and memory cells

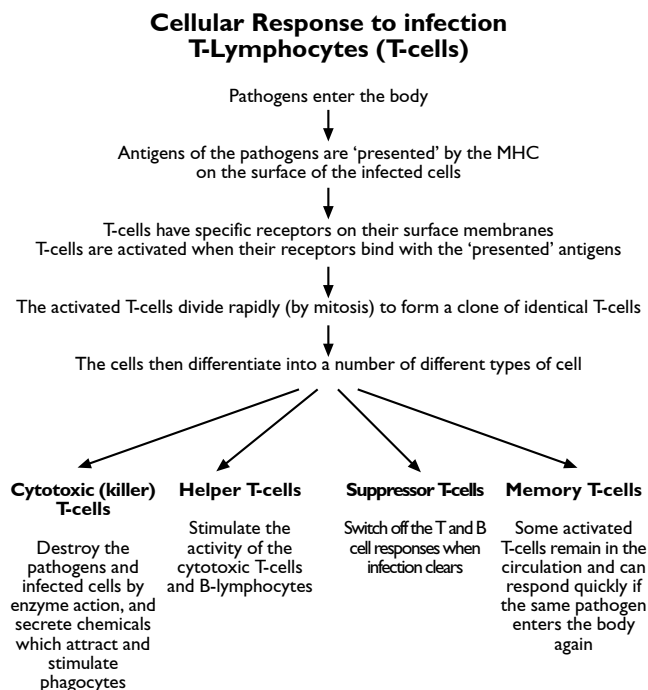


Antibody production



Immunological Responses of T-lymphocytes

The response of T-lymphocytes to foreign material differs from that of B-lymphocytes in that T-lymphocytes do not produce antibodies but work directly on infected cells. They may either lyse the cells directly by secreting enzymes or may interact with other branches of the immune system to effect their killing. As with B-lymphocytes, there are thousands of different types of T-lymphocyte each of which will only recognise one specific antigen.



ABO blood groups

In common with all human cells, red blood cells or erythrocytes have a number of proteins embedded into the cell membrane (see section 11.3), some of which are involved in cell recognition. As discussed above, such protein molecules are known as antigens or cell surface antigens and allow the immune system to distinguish between 'self' and 'non-self' cells. A number of different types of antigen occur on the red cell membrane, with the most well known being the ABO blood group system. Whilst the evolutionary significance of this genetic variation in humans is not well understood, its significance in medicine, and more specifically blood transfusion, is well established.

Group A individuals have A antigens on the surface of their red blood cells, Group B individuals have B antigens, Group AB individuals have both A and B antigens, and group O individuals have no antigens. The blood plasma of individuals of each of the four blood groups also differs in that it carries preformed antibodies against foreign ABO antigens. Thus a person of Group A will have antibodies against group B antigens (anti-B antibodies), and a person of Group B will possess anti-A antibodies. Since an AB individual has both A and B antigens on their red cells they will have neither anti-A or anti-B antibodies in their plasma. Group O individuals possess both anti-A and anti-B antibodies. As discussed above, if an antigen meets its corresponding antibody the antibody will bind to the antigen and may cause the foreign cells to clump together. Such a reaction, when blood of one ABO type is mixed with blood of another group, is known as agglutination.

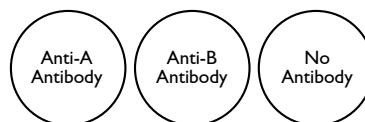
If blood of incompatible blood types are mixed in equal quantities agglutination will occur. In blood transfusions, however, generally a smaller volume of blood from the donor, is transfused into a larger volume of blood of the recipient, thus diluting the donor antibodies which are rendered relatively ineffective. Therefore in such transfusions it is only necessary for the **donor antigens** to be compatible with the **recipients antibodies**.

Blood group O is said to be the universal donor as their red cells have no antigens and can thus can be safely transfused into any individual without risk of antigen-antibody reactions. Group AB is the universal recipient, as since their plasma lacks anti-A and anti-B antibodies they can receive blood from any of the blood groups. Blood transfusions other than those indicated as 'safe' in the table below will lead to agglutination. This can cause rapid death of the patient as the agglutinated cells form blood clots which may block vital arteries within the body.

Recipient	Blood group			
Donor	A	B	AB	O
A	Safe	Agglutination	Safe	Agglutination
B	Agglutination	Safe	Safe	Agglutination
AB	Agglutination	Agglutination	Safe	Agglutination
O	Safe	Safe	Safe	Safe

Agglutination reactions noted above form the basis of blood group testing in hospitals and laboratories. Such rapid and simple tests are performed routinely in hospitals and many individuals are aware of their blood type even if they have never had a transfusion. Before an operation is begun the patient's blood type will be checked in case transfusions are needed.

Blood grouping

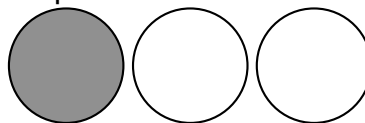


No Antibody Control

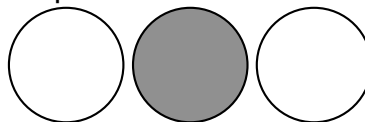
To check for contamination from area to area, and to eliminate appearance of blood clotting (which is different from agglutination) ie; if this area stays as unaltered blood the test is valid.

Drop of blood mixed in

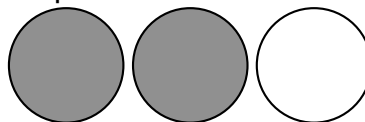
Group A



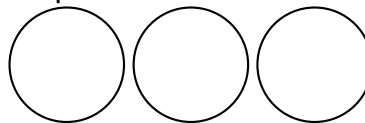
Group B



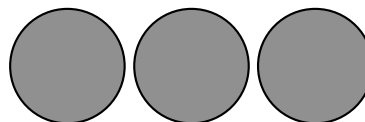
Group AB



Group O



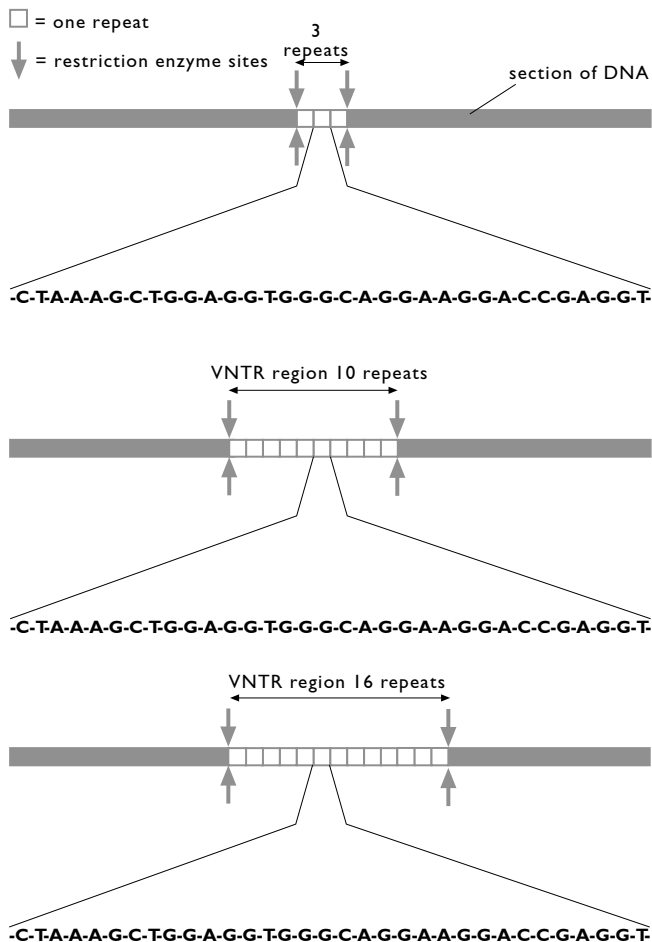
Invalid Test



GENETIC FINGERPRINTING

Genetic fingerprinting, also known as DNA profiling, is a recently developed technique which aims to reveal a specific and unique genetic pattern for each individual in the same way as each individual has a unique fingerprint. Its main application is in forensic science where, with reference to the DNA profiles of suspects or known criminals, it can help to identify the individual responsible for a crime from a small piece of DNA. It is also used in cases of disputed paternity or other family relationships. DNA profiles of organisms from other species also have applications in the fields of zoology and ecology where they can be used to assess genetic diversity and relatedness of animals (DNA profiling is expected to revolutionise the classification of organisms).

Instead of exploiting differences in genes, genetic fingerprinting relies on the extensive genetic variation which exists in the 'non-coding' DNA which lies between genes and even within genes (introns) and makes up 95% of the genetic content. Much of this non-coding DNA contains repeated sequences of sections of DNA between 15 and 100 bases long. Such regions are known as **VNTRs** (variable number of



tandem repeats) or **minisatellites**, and within them the number of repeats of a sequence is highly variable between individuals. Thus at a given site one individual may have a sequence of 17 bases (e.g. GGGGGGAACAGCGACAC) repeated 75 times, whilst another may have 200 repeats and a third person 450 repeats. The DNA sequence immediately before and immediately after the sequence will, however be identical in all individuals.

Building up a DNA profile or genetic fingerprint of an individual requires analysing the DNA of that individual at several separate VNTR sites and gathering information on the numbers of repeat sequences. Some VNTRs exist as 'families' with the same repeated sequence found at many different locations throughout the genome: these can be analysed using 'multi-locus probes' described below. Other VNTR sites occur only once and require 'single locus probes'. The high variability of the sites between people makes the process very specific: as discussed later analysis of only a small number of VNTR regions can produce a unique profile.

Restriction enzymes

Restriction enzymes or restriction endonucleases are enzymes derived from bacteria which cut DNA at specific base sequences. Such enzymes will 'recognise' particular DNA sequences known as **recognition sites**, attach to these, and cut the DNA. The process of cutting the DNA is also called 'cleavage'.

In nature, the function of restriction enzymes is to cut out sections of viral DNA which have entered the DNA of the bacterium, but in genetic fingerprinting and other genetic techniques they are used to cut a length of DNA up into smaller pieces. There are several thousand types of restriction enzymes now in use, the names of which reflect the bacterial species from which they were extracted: thus EcoRI is derived from *E. coli* and HindI from *Haemophilus influenzae*. Each restriction enzyme is specific to one base sequence and will digest or 'cleave' the nucleic acid (DNA) whenever such a site is encountered but at no other point. It should be noted that some cut straight through both strands of DNA creating 'blunt ends', whilst others produce a staggered cut or 'sticky end' which is useful in genetic engineering.

To create a DNA fingerprint of an individual the first stage is to mix a sample of DNA with restriction enzymes which cut at the two ends of one or more known VNTR sites. (Remember that such sites will have a common start and end sequence even though there are variable numbers of repeats in between.) The fragments produced will vary in length depending on the number of times the DNA sequence is repeated. It is important that there are no restriction enzyme sites within the repeat sequences as this would prevent analysis of VNTR regions on the basis of size.

Electrophoresis

This is a technique used in the analysis of DNA which has been cut into fragments of differing sizes by restriction enzymes. It is based on the principle that smaller fragments of DNA will migrate through a gel faster than larger ones when a direct electric current is applied across the gel (which is in a buffer solution). The reason for this is that the larger fragments have more difficulty moving through the

SUMMARY OF GENETIC FINGERPRINTING

- ◆ Creating a genetic fingerprint from the DNA of an individual involves a number of stages:
- ◆ Extraction of the DNA from cells (may be white cells, sperm cells in semen, hair cells etc)
- ◆ Amplification of the DNA using the polymerase chain reaction (PCR)
- ◆ Use of restriction enzymes to cut the DNA at specific sites either side of VNTR region
- ◆ Use of gel electrophoresis to split the DNA fragments up on the basis of their size (i.e. on the number of repeats)
- ◆ Southern blotting and use of radioactive DNA probe to locate the fragments of DNA
- ◆ Autoradiography to create an image of the DNA pattern for interpretation.

Enzyme	Source Organism	Restriction Site
EcoRI	<i>Escherichia coli</i>	↓ -G-A-A-T-T-C- -C-T-T-A-A-G- ↑
EcoRII	<i>E. coli</i>	↓ -G-C-C-T-G-G-C- -C-G-G-A-C-C-G- ↑
Hae III	<i>H. aegyptius</i>	↓ -G-G-C-C- -C-C-G-G- ↑
HpaII	<i>H. parainfluenzae</i>	↓ -C-C-G-G- -G-G-C-C- ↑
SmaI	<i>Serratia marcescens</i>	↓ -C-C-C-G-G-G- -G-G-G-C-C-C- ↑

dense gel. All fragments will however move, as they are negatively charged and move towards the positive terminal.

To perform electrophoresis, a gel is made from agarose (a polysaccharide) and placed in a tank of buffer solution. DNA samples that have been treated with restriction enzymes are loaded into wells in the gel along with a dye solution. This ensures that all have equal electrical charge and that their position can be marked. A direct current is passed through the buffer solution causing the DNA marked by the dye to move through the gel. Once the dye front has migrated about three quarters of the way down the gel the current is switched off. The position of DNA fragments on the gel cannot be seen at this stage: further steps are required for this.

Southern Blotting

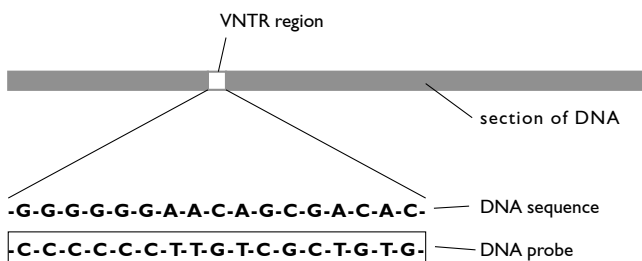
The first stage in allowing visualisation of the DNA fragments is known as Southern Blotting. There are two basic stages involved:

- ▼ firstly the DNA on the gel needs to be heated to cause the two strands of the helix to unwind making single stranded DNA;
- ▼ secondly this single stranded DNA is transferred from the gel to a nitrocellulose filter or a nylon membrane. The membrane will bear an exact copy of the gel in terms of the positions of DNA fragments. It is very important to make the DNA single stranded: if this is not done then the gene probe described below cannot bind.

DNA probes

A DNA or gene probe is a single stranded DNA sequence usually 10 nucleotides or more in length which is complementary to a stretch of known DNA (e.g. part of a repeat sequence in a VNTR region) on a chromosome. The probe is radioactively labelled using ^{32}P (this radioactive phosphorus is incorporated into the nucleotides which make up the probe). The probe is added to the nitrocellulose filter (produced via Southern blotting) where it will attach to single stranded DNA of a complementary sequence. This is known as hybridisation.

DNA in a VNTR and DNA probe



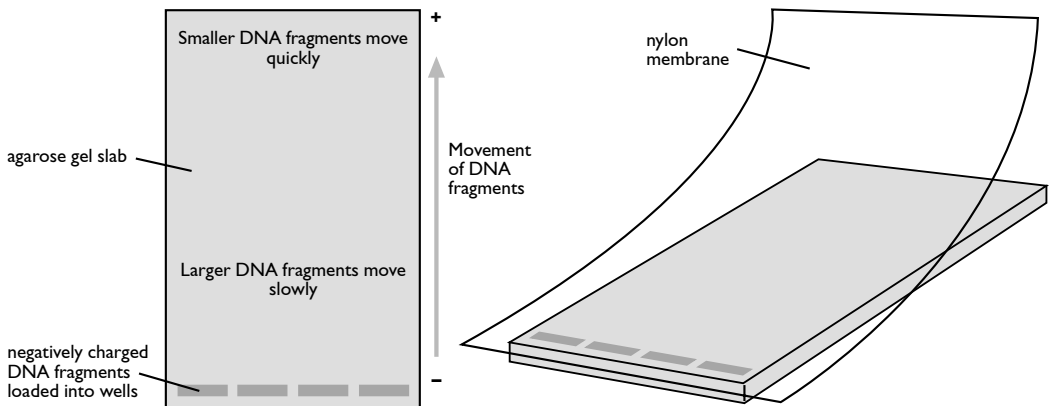
The probe will bind to any region with this sequence regardless of the number of repeats that exist. As discussed above, copies of a multi-locus probe will bind to more than one site as it has a complementary sequence that is common to several VNTR sites. A single locus probe will detect and bind to only one VNTR site in the entire DNA of an individual.

Autoradiography

In order to visualise the bound DNA probe and hence detect the size of DNA fragments separated in the original gel an X-ray film is placed over the nitrocellulose filter. At sites where the gene probe has bound to DNA fragments the radioactive P will create a black band on the film.

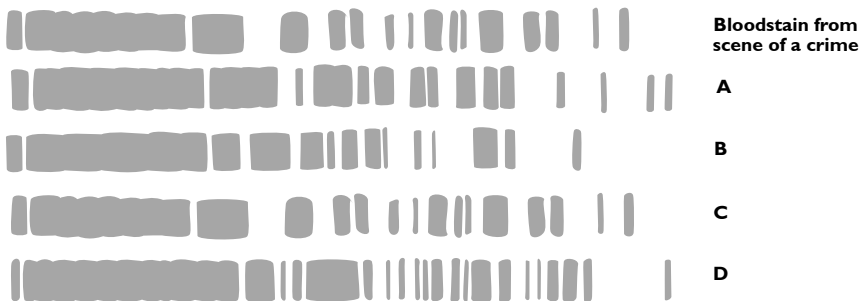
Analysis works as follows. A black band at the bottom end of the picture will correspond to small DNA fragments to which the probe has bound; i.e.. a VNTR site with a small number of repeats which has migrated a long way through the gel. Conversely black bands at the top of the picture reveal large fragments to which the probe has bound. The actual lengths of the fragments and hence the number of repeats can be compared to known lengths in marker DNA loaded into the gel. Each individual will have two bands for each VNTR which may be the same or different. Lengths of DNA are measured in kilo bases (Kb) (1 Kb = 1000 bases).

- 1 Extracted DNA e.g. from white blood cells, semen etc.
- 2 DNA cut into pieces by **Restriction Enzymes**
- 3 DNA pieces are separated by **Gel Electrophoresis** this takes 24 hours



- 4 **Southern Blotting**, the DNA fragments are transferred (blotted) onto a nylon membrane laid on top of the gel slab.
- 5 **Gene Probe Treatment** - the nylon membrane is put into a tank of single strand DNA's (probes) which are complementary to specific core DNA sequences and bind to them. The probes may be radioactive or fluorescent. Excess probes are washed off
- 6 X Ray film is placed on the membrane and the typical bands appear where the presence of **radioactive/fluorescent probes** fog the film.
- 7 The X Ray film is processed to form the **DNA Fingerprint** with its characteristic 'barcode' pattern of dark bands.

Genetic Fingerprinting - Autoradiograph bands



Was individual A,B,C or D more likely to have been present at the scene of the crime?



Which male is the child's father?

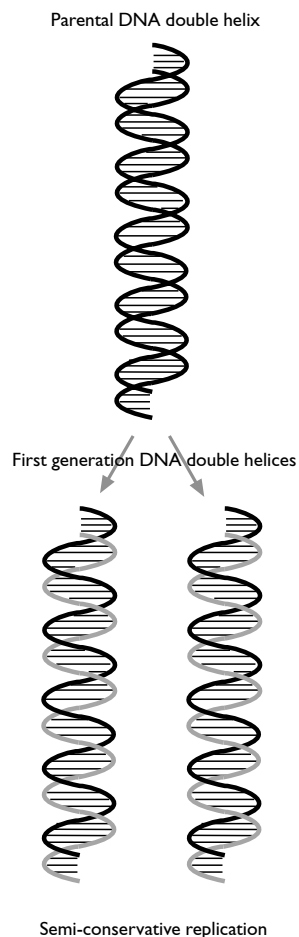
Reliability of genetic fingerprinting

As discussed above, genetic fingerprinting is used in forensic investigations where DNA collected at the scene of a crime can be analysed and compared to the genetic fingerprints of known individuals, or to fingerprints prepared from unknown individuals. For reliable comparisons several VNTR sites must be compared. It is estimated that if one single locus probe is used the same pattern of bands will occur in 1 person out of 100. But when 2 are used the number jumps to 1 in 10,000. With several probes the chances of two sets of DNA matching are so low that the test becomes highly specific in identifying individuals.

It should be noted, however, that whilst genetic fingerprinting can theoretically identify a single individual from the DNA contained in one cell it cannot stand alone in forensic or other investigations, but only as a back up to other types of evidence. For example, finding a human hair at the scene of a crime may allow you to make a genetic fingerprint of the person from whom it came. With reference to a genetic database this could allow an individual to be identified. But without other evidence this cannot be used to convict a person: the hair could have come from someone visiting the area prior to or after the crime, been 'planted' on purpose, or could have blown in. Furthermore, the genetic fingerprint obtained from DNA found at the scene of a crime may not be clear, the DNA may have partly decomposed or the sample could be contaminated.

POLYMERASE CHAIN REACTION (PCR)

PCR is a technique used to amplify (make many copies of) a DNA molecule or part of a DNA molecule. Based on an understanding of the semi-conservative mode of DNA replication in nature, PCR has revolutionised genetic technology. It theoretically allows any number of copies of DNA to be produced from a single DNA molecule (the template). Often it is used to amplify a known section of DNA only. PCR is crucial in generating copies of DNA for use in such processes as: genetic fingerprinting and genetic screening for diseases.



Amplifying a piece of DNA by PCR requires the 4 'ingredients' listed below

- ▼ DNA. Theoretically a single DNA molecule is sufficient, but usually a larger amount of DNA is used.
- ▼ Primers. These are short single stranded pieces of DNA which bind to the parent DNA strands and initiate the synthesis of a complete new strand of DNA on the template of the parent strand. One primer is needed for each parent DNA strand.
- ▼ A supply of each of the 4 nucleotides containing the bases A, C, T and G. The bases on the free nucleotides will pair with their complementary bases on the DNA parent strand (A with T and C with G) thus synthesising the new strand.
- ▼ The enzyme Taq polymerase. This enzyme works like human DNA polymerase, catalysing the attachment of the complementary bases to one another. Taq polymerase is, however, thermostable (temperature resistant) as it is derived from the bacterium, *Thermus aquaticus*, which inhabits hot springs. This is important, as PCR requires high temperatures to work.

The reaction mixture made of these 4 ingredients is placed in an individual well on a plastic plate, or in individual tubes with a layer of oil on top to prevent evaporation. The reactions involve alternate heating and cooling of this mixture and are performed by a PCR machine. The machine can be programmed for different numbers of cycles, the more cycles the more copies of the DNA are produced.

Polymerase Chain Reaction (PCR)

Allows short lengths of DNA to be copied millions of times.

Process is very rapid - each cycle takes about 5 minutes.

Four ingredients required:

A length of DNA helix to be copied;

B four types of free nucleotides;

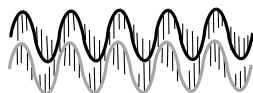
C Primers - short, single stranded pieces of DNA (about 20 bases long) complementary to bases at the start of the portion of DNA to be copied;

D DNA polymerase enzyme.

1 Original double strand DNA



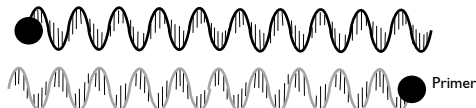
2 High temp. (96°C) strands uncoil



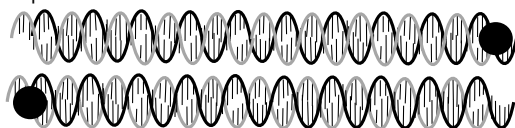
3 DNA primers DNA polymerase and nucleotides added

4 Temp reduced DNA 'Primers' attach (anneal) to the strands to be copied

Primer



5 DNA (Taq) polymerase organises free nucleotides to form complementary strands DNA is replicated



6 Process repeated many times very rapidly The original length of DNA has been amplified. Used to copy DNA from bones, blood, mummies, insects in amber, etc.

◆ SUMMARY OF THE STAGES OF PCR

For simplicity the stages of PCR are described here as if a single complete DNA molecule is used as the starting point

- ◆ PCR mixture is heated causing the DNA double helix to unwind exposing the bases
- ◆ The mixture is cooled and primers bind to the ends of each DNA strand. This is known as annealing
- ◆ Taq polymerase allows free nucleotides to pair with the exposed bases on both DNA strands. A pairs with T and C with G. Binding begins immediately after the primer sequence and continues until the whole complementary strand has been synthesised. Two new DNA double helices result. Each contains one of the original parent strands. One complete cycle has now been completed
- ◆ The process is repeated starting with two copies of the DNA and ending with four: (Each DNA molecule unwinds, has a complementary strand synthesised and winds back up). As the process is repeated over and over again the number of copies of the DNA increases geometrically: 2→4→8→16→32→64→128 etc.
- ◆ The DNA produced is used in genetic fingerprinting, genetic screening or other genetic studies. Alternatively it may be frozen and stored for future analysis.

Cultivated plants and the manipulation of the environment to increase productivity

Contents

Adaptations of cereals

- ▼ Structural and physiological adaptations to different parts of the world
 - Rice as a swamp plant with hollow aerenchyma and a tolerance to ethanol produced by anaerobic respiration.
 - Sorghum as a plant which grows in hot, dry conditions; its xerophytic modifications include the presence of an extensive root system, a thick cuticle and a reduced number of sunken stomata; both the adult plants and the embryos can tolerate high temperatures.
 - Maize as a tropical plant with a specialised method of photosynthesis; the advantages of this method of photosynthesis in increased efficiency at high temperature and low carbon dioxide concentrations.

Controlling the abiotic environment

- ▼ Human control of the abiotic environment of crop plants
- ▼ The effect of light intensity, temperature and carbon dioxide concentration on rate of photosynthesis and productivity. Enhancement of these factors in commercial glasshouses.

Fertilisers

- ▼ The use of fertilisers to replace nutrients lost by harvesting crops.
- ▼ The advantages and disadvantages of organic and inorganic fertilisers. The relationship between yield and quantity of fertiliser added. The environmental issues arising from the use of fertilisers. Leaching and eutrophication.

Pesticides

- ▼ Interspecific competition between weeds and crop plants. Reduction of crop yield by insects either directly or indirectly by reducing the photosynthetic tissues of the plant.
- ▼ The principles of using chemical pesticides, biological agents and integrated systems in controlling pests of agricultural crops.
- ▼ The environmental issues associated with pest control. Toxicity and bioaccumulation.
- ▼ Evaluation of the issues involved in using different methods to control pests

ADAPTATIONS OF CEREALS

Cereals are seed crops derived from the family of plants known as the Graminae, or grasses. They have formed the main energy providing component (staple food) of most human diets since our early ancestors turned from a hunter-gatherer existence to the collection and cultivation of selected wild grass seeds. The civilisations of the ancient world were founded on settled agricultural communities and their cereal crops; rice in the Far East, wheat and barley in the Middle East, *Sorghum* in Africa, and maize in Central and South America. All of these crops could be eaten as whole grains, ground into flour and baked into bread of some kind, or simply mixed into a porridge-like paste.

Rice

The ancestral grass plants from which rice has been developed were adapted to live partially submerged in marshy regions. It is possible to grow rice on dry land like wheat, even in semi-temperate areas but the yield increases up to three times when it is grown in paddy fields in tropical or sub-tropical temperatures. Over half the world production is in irrigated soil, notably in the great deltas of Asian rivers such as the Ganges, Irrawaddy and Mekong. Modern varieties are fast growing, taking as little as 120 days from seed to harvest, and it is common for three crops to be produced each year. For most of the growing period the rice plants are partially submerged but the land is drained just before harvest so that the dry seed can be collected efficiently.

Most plants can not survive flooding. This is because plant roots require a good supply of oxygen for aerobic respiration. Remember that the uptake of minerals is an active process requiring high energy levels. In the absence of oxygen the roots will respire anaerobically but this can not be tolerated for long because they are soon poisoned by the ethanol released as a bi-product. Rice roots have an unusually high tolerance to ethanol and their seeds can germinate in oxygen starved soils. In common with other water adapted plants they also develop an interconnecting system of large air spaces linking the roots with the leaves and other parts above water. This specialised tissue is called **aerenchyma** and it allows the flooded roots to breathe.

Rice seeds are stripped of their outer coat and embryo (the parts containing proteins and minerals) by a process called 'polishing' to produce the product which you find in the supermarkets. It is virtually pure starch and provides virtually all of the daily energy intake for over 50% of the world's population.



Maize

The ancestry of maize is uncertain. There are no modern grasses which are obviously related to it, but it is certain that the plant originated in Central America, probably in Mexico. It thrives in hot climates with well watered soils, and is adapted to photosynthesis successfully at light intensities and temperatures higher than most plants can tolerate, and with a reduced supply of carbon dioxide.

When all other conditions for photosynthesis are at their optimum level, the rate of carbohydrate production will increase with increasing light intensity until it reaches a maximum value called the **light saturation point**. At high levels of illumination the enzyme Rubisco, which normally catalyses the fixation of carbon dioxide into sugars, starts to react with oxygen in a process called **photorespiration**. Photorespiration uses up oxygen and releases carbon dioxide. It yields no useful energy and is therefore an essentially wasteful process consuming up to 25% of the carbohydrate manufactured by photosynthesis. In order to recapture the lost carbon dioxide, plants are required to open their stomata at the hottest and brightest times of day, thereby suffering a further loss of water by transpiration. This is particularly significant in tropical climates where light intensities commonly exceed the light saturation point. Maize and some other tropical plants, notably sugar cane and *Sorghum*, have evolved a mechanism referred to as **C4 photosynthesis** which overcomes the problem of photorespiration by increasing the concentration of stored carbon dioxide in their leaves. This gives them a higher light saturation point, and therefore a higher productivity.

Maize is most commonly encountered in the form of sweet corn, pop corn or tortilla chips in Western Europe. In Central and South America, maize flour is the basic material for the manufacture of tortillas, tamales and a variety of drinks. It is the single most important crop in the United States although 90% of the production is for animal feed.



Maize crop



Male flower



Female flower

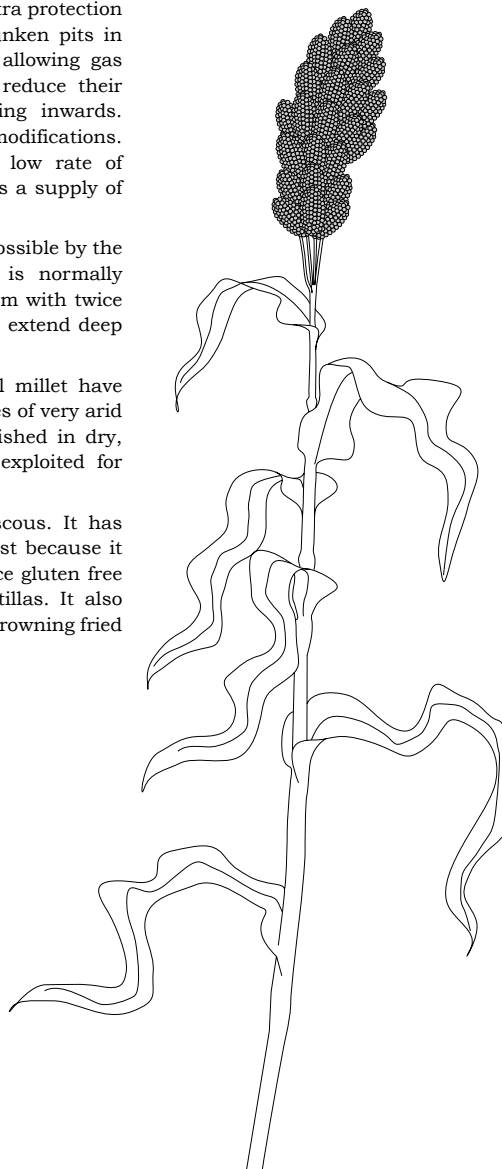
Sorghum

Like maize, sorghum is a C₄ plant, specialised to maximise photosynthetic yield in high light intensities and hot climates. Compared to maize, however, *Sorghum* is able to tolerate dry conditions, requiring 20% less water to produce equivalent yields of dry matter, and is able to grow in more hostile climates than any other cereal crop. Plants which are specially adapted to tolerate dry conditions are called **xerophytes**. The adaptations (xerophytic features) centre on ways of reducing water loss whilst retaining photosynthetic activity. Sorghum is similar to maize in its general structure but its leaves are more specialised for water conservation. They are coated with a white waxy layer, giving them extra protection from desiccation, and the stomata are situated in sunken pits in order to reduce unnecessary water loss, whilst still allowing gas exchange. In conditions of water stress, the leaves reduce their exposed surface area by becoming erect and curling inwards. Photosynthetic production is not hampered by these modifications. Sorghum retains high photosynthetic yields with a low rate of transpiration. As in maize, the C₄ mechanism provides a supply of carbon dioxide even when the stomata are closed.

Survival of grasses over extended dry periods is made possible by the possession of a much larger rooting system than is normally necessary. Sorghum has a very extensive rooting system with twice as many side branches as that of maize. Its roots also extend deep enough to extract water in drought conditions.

Sorghum and the even more drought resistant cereal millet have become the staple foods of people living on desert fringes of very arid land. These crops have also been successfully established in dry, nutrient depleted and wind eroded soils previously exploited for cotton production.

Sorghum is used to make bread, porridge and couscous. It has enjoyed a period of renewed market interest in the west because it yields a gluten-free flour which may be used to produce gluten free pizza bases, breakfast cereals and Mexican style tortillas. It also caramelises very easily and is suitable as a coating for browning fried foods.



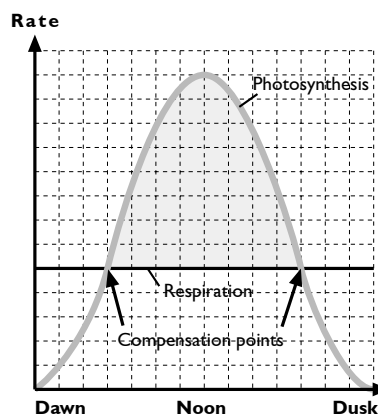
CONTROLLING THE ABIOTIC ENVIRONMENT

Human control of the abiotic environmental factors affecting productivity

A permanent increase in plant dry mass only occurs when the amount of carbohydrate produced by photosynthesis exceeds the amount oxidised in respiration and photorespiration. Remember that respiration is continuous over the whole 24 hours, whilst photosynthesis and photorespiration can only occur in the light, and it is the difference between these processes which determines plant productivity. The zero position at which the rate of photosynthesis and respiration are the same is referred to as the **compensation point**.

Productivity is measured as the gain in plant dry mass per square metre of land surface, but it is important to take into account the ratio between the leaf area of a crop and the land space it occupies. This ratio is called the **Leaf Area Index** or **LAI**, and it is calculated as the total leaf area per square metre of land. In the early stages of crop growth when new leaves are developing the LAI is small, but as more leaves are formed, it rapidly increases. Some leaves will shade each other, and in mature plants, older leaves die back as new ones are produced. For this reason it is rarely possible for a crop to use more than 95% of the available light.

Compensation points



Light intensity

The efficiency of light energy conversion into carbohydrate in crops depends not only on the amount of light received, but must also take into account losses through respiration and photorespiration (see above). The upper leaves of a plant tend to be illuminated at a level above the light saturation point for most plants. For a typical temperate crop, crop yields expressed as dry matter production relative to total incident light energy represent a less than 1% conversion of the solar energy input into chemical energy.

Productivity can be increased significantly by using crops bred to achieve high short term growth rates by maximising their percentage light utilisation. Crop research has also focussed on reducing losses by photorespiration, or increasing the availability of carbon dioxide which, under natural conditions, is normally the limiting factor.

Temperature

The ecological distribution of plants, their seasonal vegetative and reproductive cycles, and day to day carbohydrate assimilation, are all influenced, to a greater or lesser degree, by the ambient temperature. Temperature, like carbon dioxide and light, can be a limiting factor in photosynthesis, but it is more accurately regarded as an indirect influence, because above 15-20°C, it affects the rate of respiration more strongly than the rate of photosynthesis.

The most important factor affecting productivity is the average night temperature. 'Dark respiration' uses up twice as much assimilated carbohydrate at 25°C than at 15°C, for example, so cool nights may promote better growth.

Temperature has other indirect effects on the rate of photosynthesis, notably on stomatal opening but this is only in the extreme range. At temperatures above 30°C stomata generally close, probably as a result of higher carbon dioxide levels created by increased respiration.

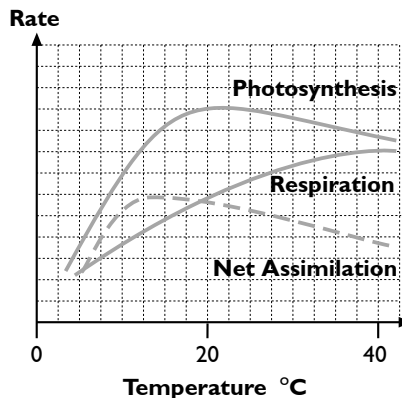
Most temperate crops give their best production between 10 -15°C, but the optimum for other regions varies from 5 °C in some arctic species to 30°C for a tropical crop like maize.

Carbon dioxide

Carbon dioxide forms a very small proportion of the atmosphere, just 0.04% by volume, but it is estimated that crop plants fix an average $160\,000\text{ kg.km}^{-2}.\text{year}^{-1}$. Although the quantity of carbon dioxide in the atmosphere remains relatively constant, local concentrations around a crop can range from 0.015-0.1%. Photosynthesis depletes the available carbon dioxide, particularly in a closed environment like a greenhouse. The most important source for replenishment comes from the respiration of soil organisms carrying out decomposition of dead organic matter (soil respiration). In a fertile soil with adequate dead organic matter and soil organisms, the carbon dioxide produced by soil respiration and released to the air will exceed photosynthetic requirements.

The amount of carbon dioxide available to crops is limited by the frequency and degree of opening of its leaf stomata and also by the **microclimate** which surrounds the individual leaves. Around each leaf a **boundary layer** of still air tends to reduce the concentration gradient for diffusion. Air turbulence is important to replace the layer of air which surrounds the leaf canopy. Windy conditions help to overcome the problem of carbon dioxide depletion in a field of photosynthesising maize in high light intensity.

Productivity (net assimilation)



Glasshouse production and Hydroponic Systems

An understanding of the various environmental factors controlling plant growth has led to the development of soil-free or **hydroponic** plant culture systems. Hydroponic systems provide a 'state of the art' illustration of how a detailed knowledge of the effects of abiotic factors can be applied to plant production. By maintaining all the limiting environmental and nutritional factors at their optimum level in controlled glasshouses it is possible to achieve a high yield in a small space and a short time period.

Hydroponic systems can be set up anywhere regardless of soil conditions and climate, even, as the Japanese have shown, within urban supermarkets to provide fresh clean vegetables with zero transport and minimum packaging costs. Pest and disease problems are minimal, and weeds do not have a chance to enter the system. With no large scale mechanical cultivation and no release of agrochemicals and fertilisers into the environment, hydroponic crops are environmentally friendly, particularly in systems where the water and nutrients are recycled.

Balancing these obvious benefits are the large set-up and skilled labour costs. It must also be considered that although disease is largely eliminated, if a crop does become infected, the process can be rapid and devastating.

The most common hydroponic system for growing tomatoes, cucumbers and lettuces is the **Nutrient Film Technique**, where plants are grown within gullies made from plastic film. Typically, the gully is constructed from a sheet of plastic, 65-80cm wide, coated black on the inside and white on the outside. It is stapled together between the plants and gently inclined so that the nutrient solution drains into catchment pipes which re-circulate it via a catchment tank and associated pumping system.

If plant roots require extra aeration, the nutrient solution may be supplied intermittently (in bursts).

Problems may arise when vigorous root growth prevents circulation of fluids.

◆ CHECKPOINT SUMMARY

- ◆ Productivity is measured as the gain in plant dry mass per square metre of land surface. This reflects the relative rates of photosynthesis and respiration
- ◆ The abiotic environment includes light intensity, temperature, and carbon dioxide concentration
- ◆ Increasing light intensity increases the rate of photosynthesis up to a certain point, the light saturation point, past which there are no further gains as the result of light stimulated photorespiration
- ◆ Temperature, like carbon dioxide and light, can be a limiting factor in photosynthesis, but it is more accurately regarded as an indirect influence, because above 15-20°C, it affects the rate of respiration more strongly than the rate of photosynthesis
- ◆ The most important factor affecting productivity is the average night temperature. 'Dark respiration' uses up twice as much assimilated carbohydrate at 25°C than at 15°C, for example, so cool nights may promote better growth
- ◆ Most temperate crops give their best production between 10 -15°C, but the optimum for other regions varies from 5 °C in some arctic species to 30°C for a tropical crop like maize.

Controlling the glasshouse environment

Seasonal and climatic changes in light intensity, wavelength and duration may be compensated for in a variety of ways. Where day length influences flowering, e.g. chrysanthemums, it is controlled by the use of artificial light and/or partial shading.

The optimum temperature for tomatoes and lettuces is 18–22°C. It is important to lower the night temperature by 6–12°C in order to minimise dark respiration. Control is achieved through thermostatic monitoring and the use of heaters, vents, shades and, where necessary, coolers. The temperature directly affects humidity so fine mist sprays are linked to the same control system.

Glasshouse plants, being confined to an enclosed space, quickly use up the available carbon dioxide and so it is very important to supply this by some external means. Where heating is the norm, a hydrocarbon burner may be used to supply carbon dioxide. Alternatively, dry ice or gas cylinders may be used.

Insect pests are potentially disastrous in the glasshouse environment. They are sometimes controlled by pesticides introduced via the fine mist spray system, although biological control agents are increasingly common. In the absence of chemical insecticides, bee hives may be kept in glass houses providing natural pollinators for fruits such as tomatoes, cucumbers, and strawberries. Otherwise pollination must be achieved manually.

FERTILISERS

Crop productivity is affected by the availability of inorganic nutrients in the soil. Plants take up inorganic elements from the soil solution in the form of anions (negatively charged ions) and cations (positively charged ions). Some elements, like nitrogen, phosphorus, and potassium are required in relatively large quantities (up to 150 kg per hectare). These are called **macronutrients**. Others such as zinc and copper are needed in trace amounts only and these are termed **micronutrients**. Some make up structural components of plant cells; nitrogen, for example, is essential for amino acid synthesis, phosphorus occurs in DNA and ATP, and magnesium forms part of the chlorophyll molecule. Other elements such as potassium are involved in enzyme systems. Iron is a component of the cytochromes which make up the respiratory electron transport chain; calcium controls membrane permeability, preventing leakage of other ions from plant cells.

The mineral nutrients most frequently applied to soils as fertiliser are nitrogen, phosphorus and potassium. They are often combined in what is known as an **NPK mixture**. The relative amounts of each component and the timing of the application can influence both the development and yield of a crop. In a maize crop, for example, nitrogen is important at the end of the growing season when the protein content is being laid down in the seed.

Nitrogen is the most important limiting nutrient because without it, plants cannot make proteins. Nitrogen exists in the soil as nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) ions. All of these can be directly utilised by plants but by far the most commonly available form is the nitrate ion (NO_3^-). Nitrate is released as a result of the activity of nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* which oxidise ammonium compounds utilising the energy derived from these reactions to manufacture organic food compounds.

◆ CHECKPOINT SUMMARY

- ◆ The quantity of carbon dioxide in the atmosphere remains relatively constant at 0.04%, but local concentrations around a crop can range from 0.015 - 0.1%, and under natural conditions in daylight carbon dioxide is the rate regulating (rate limiting) factor for photosynthesis
- ◆ By maintaining all the limiting environmental and nutritional factors at their optimum level in controlled glasshouses it is possible to achieve a high yield in a small space and a short time period
- ◆ An understanding of the various environmental factors controlling plant growth has led to the development of soil-free or hydroponic plant culture systems.
- ◆ Where day length influences flowering as in the case of chrysanthemums it is controlled by the use of artificial light and/or partial shading
- ◆ The optimum temperature for tomatoes and lettuces is 18–22°C. It is important to vary the day and night temperature by 6–12°C in order to minimise dark respiration. The temperature directly affects humidity so fine mist sprays are linked to the same control system
- ◆ Glasshouse plants, being confined to an enclosed space, quickly use up the available carbon dioxide. Where heating is the norm, a hydrocarbon burner may be used to supply carbon dioxide. Alternatively, dry ice or gas cylinders may be used.

Nitrate ions (being negatively charged) are not held by the negatively charged clay particles in the soil, and are very susceptible to being washed out of the soil (leaching). This causes variations in the soil concentration even on a daily basis. By contrast, ammonium ions (NH_4^+) are held by the negative charges on soil particles and are directly usable by plants.

Phosphorus is an essential component in three major biomolecules, ATP, nucleic acids and phospholipids. It is taken up by plants in the form of H_2PO_4^- or HPO_4^{2-} ions. These ions do not tend to move freely in the soil and become fixed in compounds not available to plants. Up to 80% of phosphate fertiliser, normally applied in the form of 'super phosphate' (P_2O_5), can be wasted in this way, so it is sometimes introduced with the seed as it is sown, a practice called **banding**, ensuring that the nutrient is available where the roots actually grow.

Potassium is absorbed in large quantities by plant roots, reaching concentration levels up to ten thousand times that of the soil solution in some *Brassica* (cabbage and rape) species. Potassium ions have a role as activators in enzyme systems, notably in protein synthesis, and they play a key role in the opening and closing mechanisms of stomata.

Organic and inorganic fertilisers

Organic fertilisers should be seen as complementary to, rather than an alternative to, the inorganic (chemical) fertilisers described above. The main source of organic nutrients is animal waste, either in solid form mixed with bedding materials (farmyard manure, FYM) or as liquid or semi-liquid (slurry). The quality of animal manure depends on the type of animal, what it is fed on, and how much bedding is used. It is ploughed into the soil, yielding a slow release of nutrients. More importantly, it improves soil structure by binding small particles together and retaining water.

Modern intensive animal farming methods tend to use less bedding, and mechanical devices shift the dung to storage tanks in liquid or semi-liquid form. Liquid manures are potentially very hazardous to the environment. They have a high biological oxygen demand (BOD) causing eutrophication, and far from improving soil structure, they tend to create anaerobic conditions by flooding the soil air spaces. For these reasons strict regulations apply to their use where flooding may occur near rivers.

Other sources of organic nutrients include sewage sludge, seaweed, waste material from the cotton and wool industries and dried blood and bone meal. Sewage sludge whilst cheap and easily available, may contain pathogens and heavy metals and is best avoided altogether. The use of other sources depends upon the relative local costs and benefits.

Green manure

Green manure is the term used to describe the practice of growing and ploughing into the soil a green (non-seed) crop such as white mustard or clover. Green crops may be used as 'cover crops' which cover the soil during the winter months, preventing the erosion and leaching of valuable inorganic nutrients.

◆ CHECKPOINT SUMMARY

- ◆ Harvesting removes nutrients from the soil by preventing the nutrients in the crop from returning to the soil as a result of decomposition
- ◆ Crop productivity is affected by the availability of inorganic nutrients in the soil, which are supplemented by the addition of fertilisers
- ◆ The mineral nutrients most frequently applied to soils as fertiliser are nitrogen, phosphorus and potassium. They are often combined in what is known as an NPK mixture
- ◆ Nitrogen is the most important limiting nutrient
- ◆ Up to 80% of phosphate fertiliser; normally applied in the form of 'super phosphate' (P_2O_5), can be wasted by becoming fixed in compounds not available to plants
- ◆ Organic fertilisers should be seen as complementary to, rather than an alternative to, the inorganic (chemical) fertilisers described above. The main source of organic nutrients is animal waste
- ◆ Organic fertilisers improve soil by binding small particles together and retaining water
- ◆ In modern intensive animal farming methods the dung is stored in tanks in liquid or semi-liquid form. Liquid manures are potentially very hazardous to the environment. They have a high biological oxygen demand (BOD) causing eutrophication and tend to create anaerobic conditions by flooding the soil air spaces
- ◆ Green manure is the term used to describe the practice of growing and ploughing into the soil a green (non-seed) crop such as white mustard
- ◆ The main nutrient which leaches out of the soil to pollute rivers and lakes is nitrate, which acts as a fertiliser in the aquatic ecosystem causing 'super feeding' or eutrophication
- ◆ Eutrophication causes unnaturally rapid growth of plants, occasionally forming algal blooms on the surface of the water
- ◆ The increase in plant material, over a period of time leads to increased amounts of dead organic matter which become food for aerobic bacteria. Bacteria have a high biological oxygen demand (BOD) and consequently starve the other oxygen uses of essential supplies. The highest oxygen users, notably fish and certain insect larvae suffer population decline and extinction
- ◆ In extreme cases conditions become anaerobic and the ecosystem collapses.

Leaching and Eutrophication

The main nutrient which leaches out of the soil in any quantity to pollute rivers and lakes is nitrate. The effect of nitrate in aquatic ecosystems is to fertilise the water causing 'super feeding' or **eutrophication**. This causes unnaturally rapid growth of plants, occasionally forming **algal blooms** on the surface of the water. The increase in plant material, over a period of time leads to increased amounts of dead organic matter which become food for aerobic bacteria. Bacteria have a high biological oxygen demand (BOD) and consequently starve the other oxygen users of essential supplies. The highest oxygen users, notably fish and certain insect larvae suffer population decline and extinction. In extreme cases, especially in lakes and ponds, conditions become anaerobic and the aquatic ecosystem collapses. In running water there is an 'oxygen sag' immediately downstream of the source of pollution, which slowly recovers further downstream. The quantity of nitrate applied as fertiliser to the soil should be calculated on the basis of **optimum use** by the crop and not simply on maximum crop yield.

The CONTROL of BIOTIC FACTORS (PESTS and COMPETITORS)

Weeds

Weeds compete aggressively with crops for water, light and mineral nutrients and generally show better tolerance to limited supplies. They are characterised by high rates of photosynthesis, and a rapid development of roots and leaves, so they gain access to their needs more quickly than their neighbours. Weeds tend to be the first colonisers of waste and disturbed ground, more resistant than other plants to environmental extremes, and more tolerant of changing conditions. Some, like couch grass, secrete toxic or growth retardant substances called **allelochemicals** to fight off the competition.

It is very difficult to isolate the specific effects of individual weeds in a crop because several weed species compete with the crop, and with each other simultaneously for light, water, and minerals, and the resources being competed for, are often in short supply (limiting), and interrelated. One principle, however, is universal. The closer the requirements of crop and weed, the more damaging the competition, and the more difficult it will prove to control the weed. This is most obvious when the weed and crop belong to the same plant family, for example, wild oat (*Arvena fatua*) in cereal crops, fat hen (*Chenopodium album*) in sugar beet and charlock (*Sinapsis arvensis*) in oilseed rape. If the relationship is very close there is a further danger of hybridisation between weed and crop to produce an even stronger competitor.

The degree of competition between weed and crop depends largely upon the relative timing of their vegetative and reproductive cycles. Harvest yields will be affected most seriously if the growth and reproductive stages of the weed coincide with those of the crop.

Insects

The success of insects as competitors for human food may be explained by two main biological features. One is the hard exoskeletal material, **chitin**, which can be fashioned into so many different feeding accessories that no plant part is immune from attack. Consider the mandibles and maxillae of the locust and aphid. In the locust, the mandibles form pincer like cutting tools whilst the maxillae are designed to guide a leaf blade into position for biting. In the aphids, both the mandibles and maxillae are sharpened and lengthened into a piercing hypodermic structure which penetrates the tissues of plants gaining direct access to the sugary sap in the phloem. The other biological feature of insects which accounts for their phenomenal success is their ability to survive and multiply rapidly in the harshest of conditions.

Insecticides - the Chemical Approach to Control

A general distinction can be made between chemicals which act by penetrating the outer cuticle of insects, termed **contact insecticides**, and those which are ingested, the so-called **stomach poisons**.

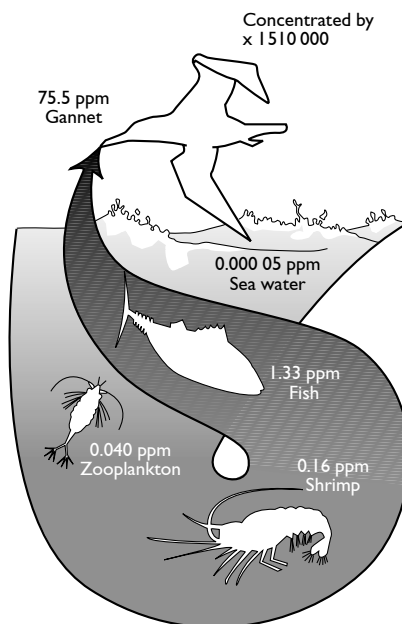
Of the older pesticides, Pyrethrum is a natural product extracted from several species of chrysanthemum (although synthetic versions have been developed) and is a contact insecticide which penetrates the cuticle of the insect as it rests against a sprayed surface.

Paris green (calcium acetoarsenite), was the first chemical insecticide used successfully on a large scale, is a stomach poison which is ingested as the insect feeds on sprayed vegetation.

Organochlorines were first developed as pesticides in the 1930s. They combine two important properties. They are **persistent**, remaining in a stable and active form for periods exceeding a year, and **residual**, accumulating in the fatty tissues without being excreted or broken down. Organochlorines destabilise the nerve membranes and tend to inhibit the respiratory enzyme cytochrome oxidase.

DDT was the first to be highlighted as having adverse effects on higher organisms quite unrelated to the target pest. The persistent and residual properties which made it so effective in eradicating the malarial mosquito and combating the insect pests of fruit trees, caused it to accumulate in natural food chains, particularly aquatic ones. Birds, such as pelicans and eagles, at the top of long food chains, diminished in number as the multiplied doses of DDT affected the shell forming mechanism in their reproductive tracts.

Organophosphates were first developed in the 1940s as poison gases for use in the Second World War. They include **parathion** and **malathion** which are still among the most widely used of all insecticides. They are easily formulated into sprays which coat the surface of leaves, and may penetrate into the epidermal tissues giving a **semi-systemic** action. When ingested by an insect pest, organophosphates act by combining with, and inactivating, the enzyme cholinesterase at nerve synapses. Cholinesterase is responsible for the removal from the synapse of the transmitter substance acetylcholine after each nerve impulse, so the result of organophosphate ingestion is the accumulation of acetylcholine in the synapses, leading to paralysis.



What are the alternatives? There are three other important approaches, namely biological control, cultural control, and the development of resistant crops, none of which alone forms a viable strategy for maintaining food levels. They should not be considered as alternatives to each other but as complementary strategies in a balanced and integrated approach to pest management.

Biological Control

Biological control involves the use of other organisms, usually predators or parasites, to reduce the numbers of a pest population. A good example is provided by the tiny parasitic wasp, *Encarsia formosa*, which has been used to control whitefly in commercial glasshouses since 1926.

Whitefly (*Trialeurodes vaporariorum*) is a sap sucking insect closely related to aphids, which owes its name to an opaque layer of wax coating its body and wings, giving it a white appearance. It is not a native British species, having been accidentally introduced from tropical America, but it thrives in glasshouses, and is very destructive of tomato, cucumber and chrysanthemum crops, not least because of its ability to transmit viral infections.

The tiny wasp, also tropical in origin, lays its eggs in young immature whiteflies referred to as 'scales' killing them before the young wasps eventually hatch out. The wasp was bred extensively for sale to glasshouse growers throughout the 30s and 40s but was made largely redundant by the advent of DDT. Biological control of whitefly using *Encarsia* enjoyed a revival in the 1970s as problems of pest resistance to chemical insecticides were recognised.

The development of a new biological control agent starts with a research programme to identify, collect and breed natural pest enemies in their natural habitat. Once sufficient numbers have been bred (and here it is important to get as much genetic variety as possible) the agent undergoes a quarantine phase in which it is assessed in laboratory conditions for its reproductive rate, climatic resistance, appetite for its pest prey, and potential threat to other organisms. After this it must undergo five years of field trials. The total cost of development is less expensive than the development of a new insecticide. Biological agents also have the advantage of reaching their target organisms in all conditions and in places inaccessible to sprays. They are kept reproducing once established in the field, and there is little chance of the pest developing resistance.

However, there are a number of problems associated with the use of biological control methods. In order to maintain a healthy breeding population of *Encarsia* it is necessary to keep the temperature of the glasshouse within very strictly controlled limits. If it is too cool, then the wasps will not breed effectively. Conversely, in warm conditions the wasps become overactive and wipe out all the young whitefly, thereby denying the next generation of its sole food supply.

You can see from this example how biological control programmes depend on carefully managed population dynamics. Despite the checks referred to earlier, there are also environmental risks resulting from a possible switch by the predator or parasite to alternative and beneficial insect prey species. New diseases are constantly imported with non-native plant introductions, many of them notifiable under strict government regulations. The penalties for delay in treatment are high, so growers are encouraged to seek rapid chemical remedies.

◆ SUMMARY OF CHEMICAL CONTROL

The chemical control of insect pests poses four well publicised hazards:

- ▼ Insecticides may be directly poisonous (toxic) to man
- ▼ Insecticides may accumulate in food chains and have serious, even fatal, effects on top carnivores (including man)
- ▼ Insecticides may reduce populations of beneficial insect species.
- ▼ The use of insecticides encourages the increase of resistance in pest species.

Cultural Control

In uncultivated land, plants, insects, and fungi co-exist within a balanced ecological system. Population numbers fluctuate, but no individual organism acquires the status of a pest. It is important to recognise that pests are created by agriculture, and the problem becomes more acute the more intensive the methods employed.

The practice of growing crops in single stands or **monocultures** accelerates the growth of particular insect populations. In uncultivated land, insects are scattered as widely as their host plants. Their populations tend to reach equilibrium at much lower densities because they encounter a greater diversity of natural enemies, and compete with each other more acutely. Intensive farming both eliminates many natural enemies, and ensures a plentiful food supply. Under such conditions, the pest population soars rapidly, reaching artificially high densities.

Cultural control is the term used to describe the application of different techniques of cultivation with the aim of reducing yield loss through pests and competitors.

Where crops are widely spaced, it is advantageous both for weed control and for moisture retention to provide ground cover between the plants. In small banana plantations this is done by **mulching** which involves covering the exposed soil surface after tillage with rotting banana leaves. A more high-tech (and costly!) version of mulching can be seen in 'pick-your-own' strawberry farms, where plastic sheeting is used to cover the ground between the rows of strawberries. A better option altogether is to fill the spaces by planting a secondary crop, for example cowpeas in maize, or groundnuts in coffee. This practice, called **intercropping** is both economically and environmentally sound.

Crop rotation is an obvious method of cultural control of insect pests. It is calculated that the maize, wheat, red clover rotation harbours up to 50 insect pest species, but no more than 3 are common to all three crops. This may be combined with the planting of mini-hedgerows, a practice referred to as **strip farming**, providing wild vegetation which will encourage the growth of natural predator populations. An ingenious method of cultural control has been used in Canada to protect wheat crops against a stem-boring sawfly. A small strip of brome grass is planted around the crop and this attracts the egg-laying female flies to deposit their cargo before reaching the wheat. When the young larvae hatch out, they tend to eat each other and little damage results to the crop. The brome grass acts as a decoy or **trap crop**.

Hygiene

Regardless of cultural practices or chemical control methods, a high degree of preventative hygiene is essential in order to avoid contamination of a crop with weed seed or fungal spores brought in on machinery or irrigation water. This is particularly important in the battle against fungal diseases. Machinery and storage facilities are disinfected after each harvest, and crop debris should be cleared, either by ploughing in or, ideally, by burning.

Integrated Crop Management

Attitudes to pest control have changed considerably over the last thirty years, not simply because of a wider consciousness and concern for environmental protection. The main change is a philosophical one, focussed by the rather late realisation that it is not possible to eliminate pest species. Instead of searching for the ultimate weapon, therefore, a more rational approach would concentrate on ways of managing pest populations.

Pest management depends upon systematic and long term evaluation of the biology of weeds, insects, and fungi, and that of their natural enemies. Computerised predictive models of infestation and crop yield assessment may be devised, and linked to weather information giving farmers early warning of potential economic damage. In this way, all the methods outlined above may be brought into play in a more coordinated manner, ensuring that war is only waged in cases where the cost of control is less than the cost of the loss in yield.

However, a new age and an additional defensive strategy has opened up with the development of transgenic resistant plants. It is now increasingly possible to manipulate all aspects of the crop, including crop protection.

Integrated crop management embraces chemical, biological, and cultural control, as well as the manipulation of plant genetics. The environmental and public health impact of any particular strategy must be assessed, both in isolation and in combination with other factors. Education of food consumers is also very important. Informed customers no longer accept indiscriminate spraying of fruit and vegetables with chemical pesticides, and they already tend to reject products from countries where such practice is common, regardless of attractive qualities such as the colour, size, price, and shelf life of the product.

◆ CHECKPOINT SUMMARY

- ◆ Weeds compete with crops for water, light and mineral nutrients and show better tolerance to limited supplies. They have high rates of photosynthesis, and development, so they out compete crop plants
- ◆ Weeds tend to be the first colonisers of cultivated ground, more resistant than other plants to environmental extremes, and more tolerant of changing conditions
- ◆ The closer the requirements of crop and weed, the greater the competition, and the more difficult to control the weed
- ◆ If related there is a further danger of hybridisation between weed and crop to produce an even stronger competitor
- ◆ Harvest yields are affected most if the growth and reproductive stages of the weed coincide with those of the crop
- ◆ The success of insects as competitors for human food may be explained by the wide variety of feeding methods, and their ability to survive and multiply rapidly in the harshest of conditions
- ◆ Contact insecticides penetrate the exoskeleton of insects, and stomach poisons are swallowed by the insect
- ◆ Organochlorines are persistent, remaining in a stable and active form for periods exceeding a year, and residual, accumulating in the fatty tissues without being excreted or broken down
- ◆ Organochlorines destabilise the nerve membranes and tend to inhibit the respiratory enzyme cytochrome oxidase
- ◆ The chemical control of insect pests poses four well publicised hazards. Insecticides may be directly poisonous (toxic) to man. Insecticides may accumulate in food chains and have serious, even fatal, effects on top carnivores (including man). Insecticides may reduce populations of beneficial insect species. The use of insecticides encourages the increase of resistance in pest species
- ◆ Biological control involves the use of other organisms to attack the pest, cultural control involves cultivation techniques which reduce conditions favourable to the pest, and the development of resistant crops involves the breeding-in of resistance to the pest
- ◆ None of these alone forms a viable strategy for maintaining food levels
- ◆ They should not be considered as alternatives to each other but as complementary strategies in a balanced and integrated approach to pest management.

Biotechnology and the manipulation of reproduction in humans and domestic animals

Contents

Reproduction and its hormonal control

- ▼ The development of ovarian follicles and corpora lutea and changes in the uterine endometrium during the sexual cycle in a female mammal
- ▼ The hormonal control of the female sexual cycle in a mammal
- ▼ The roles of FSH, LH, oestrogen and progesterone
- ▼ The detection and significance of oestrus in a named farm animal

Manipulation and control of reproduction

- ▼ The use of extracted and synthetic hormones as contraceptives and in controlling human infertility
- ▼ In domestic animals, the role of hormones in:
 - producing large numbers of embryos for transplanting
 - synchronising breeding behaviour in sheep
 - increasing milk production
- ▼ The moral and ethical issues associated with using biotechnology to manipulate reproduction

REPRODUCTION and its HORMONAL CONTROL MANIPULATION and CONTROL of REPRODUCTION

Introduction

In both male and female mammals all aspects of sexual reproduction, including gamete production and mating behaviour, is under hormonal control. In both sexes interactions occur between reproductive hormones produced by the pituitary gland under the brain, and those produced within gonads (ovary and testes) which function to ensure optimum fertility. Understanding these interactions, particularly within the female, has provided many opportunities for human intervention in the process of reproduction.

Sexual cycles in the female mammal

In the sexually mature female mammal regular sexual cycles occur, during which one or more female gametes (ova, singular ovum) develop inside follicles and are released from the ovary, offering the chance of fertilisation if mating occurs. The general term for such cycles is the oestrus cycle. Oestrus cycles really consist of two closely linked cycles which are synchronised by hormonal interactions. These are:

- ▼ the **ovarian** or follicular cycle in the ovary which is concerned with the development and release of ova.
- ▼ the **uterine** cycle which is concerned with the development of the endometrium (uterus lining) to ensure the highest chance of implantation of a fertilised ovum.

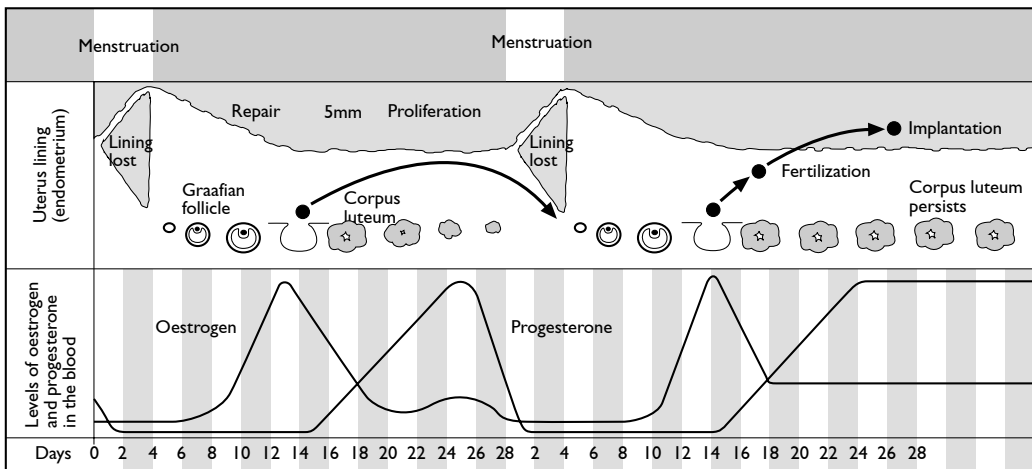
In the human female each cycle is approximately 28 days long. Ovulation which usually involves the release of a single ovum, occurs around the 14th day. The endometrium reaches a peak of thickness on day 21 and begins to break down on around day 28. This tissue, consisting of blood and cell debris is lost via the vagina during a menstrual period (menstruation). One cycle follows directly on from another throughout the year assuming that fertilisation does not take place.

Hormonal control of the oestrus cycle

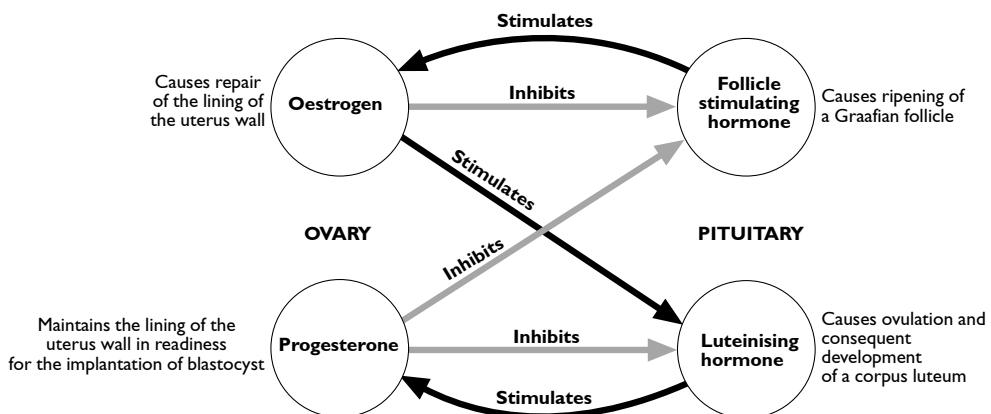
The sexual cycle relies on interactions between four main hormones: the ovarian hormones **oestrogen** and **progesterone** and the anterior pituitary hormones **Follicle Stimulating Hormone** (FSH), and **Luteinising Hormone** (LH). Although timings of the cycle vary between species the same basic pattern holds true. This can be summarised as follows.

- a) FSH is released from the anterior pituitary and travels in the bloodstream to the ovaries where it stimulates the development of several follicles (primary oocytes). One or more of these will eventually mature into an ovarian (Graafian follicle) containing the ovum (secondary oocyte)
- b) FSH stimulates the production of oestrogen from the follicles within the ovaries. This oestrogen in turn stimulates the proliferation of the uterus lining (endometrium), replacing cells lost during the previous menstrual period in humans (or, in the case of animals, those cells which were broken down and absorbed). At this point oestrogen inhibits the secretion of FSH (negative feedback control).
- c) Oestrogen levels increase until the mid point of the cycle when they bring about a peak of LH production from the anterior pituitary causing ovulation (the release of the ovum (pleural ova) secondary oocytes from Graafian follicles). A small peak of FSH production also occurs at the time of ovulation.
- d) LH causes the empty Graafian follicle to develop into a corpus luteum which begins to secrete progesterone and oestrogen.
- e) Progesterone stimulates further development of the endometrium, in particular an increase in the density of blood vessels and the secretion of mucus and fluid from glands in the lining. This is sometimes known as the secretory phase and its function is to prepare the uterus in case the newly released ovum is fertilised.

- f) The other function of progesterone is to inhibit the production of both FSH and LH and hence the development of further follicles.
- g) If fertilisation does not occur the corpus luteum degenerates and secretion of progesterone and oestrogen falls. This fall causes the uterus lining and some of its blood vessels to break down. In humans this is lost during menstruation, whilst in other mammals it is reabsorbed. Decline in progesterone levels causes the inhibition of FSH and LH to be removed and a new cycle can begin.
- h) If fertilisation does occur then the corpus luteum does not degenerate but continues to secrete progesterone and oestrogen, maintaining the uterus lining and preventing further follicles from developing. After about 12 weeks of pregnancy in humans and corresponding times in other mammals the developing placenta takes over the secretion of progesterone and oestrogen.



Oestrus cycle



Timing of sexual cycles

The length and details of the oestrus cycle varies amongst other mammals. Cycles may be as short as 4-5 days in the rat, extending to 21 days in the cow. In many wild animals, such as deer, only one cycle occurs per year which is associated with a particular breeding season probably triggered by environmental factors including changes in day length (photoperiod). Other animals with distinct breeding seasons such as the sheep may have several cycles within a given season whilst some such as cows and pigs resemble humans in having continuous cycles. This is known as being polyoestrus. Many large mammals (cow, horse) resemble humans in the release of a single ovum per cycle, whilst others such as the cat and rat may release as many as ten.

Oestrus and its significance

Oestrus is a change in the behaviour or appearance of a female mammal which occurs around the time of ovulation, indicating sexual receptiveness to males. It may involve the secretion of chemicals known as pheromones, swelling or change in colouration of the genital region or distinctive behavioural patterns, as discussed below. The human sexual cycle shows several unusual features which distinguishes it from the cycles of other mammals. Chief amongst these is the absence of oestrus, humans have the unusual feature of being sexually receptive at all times, regardless of the phase of the cycle.

In farm animals, such as cows, pigs and sheep, the presence of particular signs of oestrus which are detectable by humans, allows intervention in the process of reproduction. This is of great significance in modern farming where efficient breeding programmes are needed to allow the farm to exist as a profit-making business.

For a variety of reasons, including economic and safety reasons, many farms do not own male animals such as bulls, rams, stallions, or boars. Instead they may pay for the services of a male belonging to another individual, or, increasingly commonly, they may buy in semen with which to artificially inseminate the female animals. The use of semen (which is collected and stored in carefully controlled conditions to ensure high sperm quality and absence of sexually transmitted diseases) has many advantages over the use of natural breeding with a male animal. One of the most significant advantage is the ability to select and obtain semen from a male animal whose offspring are known to have specific desirable qualities such as high milk yield and quality, high meat quality, attractive appearance, and docility.

If artificial insemination (A.I.) or indeed mating, is to be used effectively then a farmer must be sure that the female animal is in oestrus before it is attempted. Insemination at other times will not result in pregnancy.

Signs characterising oestrus in the cow, pig and sheep are listed below. Careful observation by farmers, often several times per day, is necessary to spot these signs. Individual animals differ in the extent to which signs of oestrus are shown: some will show no signs, whilst others will show some or all of the signs.

The Cow

- ▼ Cows attempting to mount other females and then standing still and allowing themselves to be mounted by other females is the best sign of oestrus. On farms this may be detected in the following way: a type of paint is applied to the top part of the tail which will be rubbed off if the cow is mounted by another cow.
- ▼ Increased excitability. The cow shows a restless pattern of behaviour with increased time spent walking around and grooming other cows and decreased time resting.
- ▼ The cow may make a bellowing noise.
- ▼ A swollen vulva

NB. A bull may detect oestrus by sniffing the genital region of the cow (he detects chemical signals). In response to this he will show a lip curling response known as the 'flehmen response'.

The pig (sow)

- ▼ The most reliable sign of oestrus is the demonstration of the 'standing reflex' when pressure is applied to the lower back of the sow by the farmer. This is a rigid posture with the back legs extended that a female would adopt during mating. It occurs most frequently when pressure is applied to the back in the presence of a boar or when the female can detect the sound or scent of a boar. Synthetic 'boar odour' aerosols and tapes of boar sounds can be used instead of a real boar.
- ▼ A red swollen vulva and production of clear mucus from the vagina
- ▼ Sniffing of the genitals of other pigs and making a roaring or grunting noise may also occur.

The Sheep (Ewe)

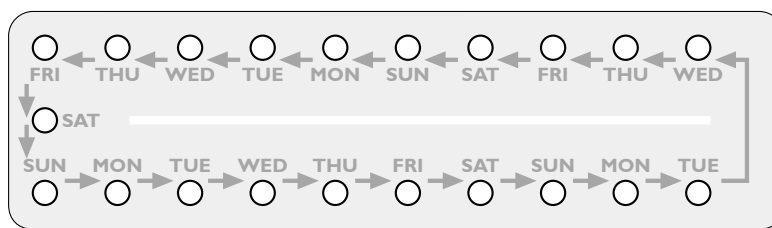
Detection of oestrus in the ewe is difficult since the sheep will only show signs if a ram is present. A farmer must therefore place a ram in with the sheep and watch the behaviour of both ram and ewes carefully. This ram may be a castrated animal and not therefore the male to be used for mating purposes. Under these circumstances oestrus is signalled by:

- ▼ Ewe standing firm and looking backwards, sometimes 'fanning' her tail (moving tail rapidly from side to side).
- ▼ Ram sniffing the genital region of the ewe, (suggesting the presence of chemical signals), curling his lip (flehmen response), pawing the ground and emitting a low pitched gurgling sound.

The control of reproduction: fertility and infertility.

The past century has seen a dramatic decline in the fertility of populations in most parts of the developed world and more recently within many developing countries. Fertility, in population terms, refers to the number of live births per 100 000 people and reflects the fertility of individual couples within that population. In Britain today each couple has, on average, 2 children compared to 6 or 7 a century ago. The main reasons for this is are an increase in the availability of contraception, associated with improvements in health (high chance of children surviving), education, and income.

Contraception can be defined as the prevention of conception (fusion of male and female gametes). Of the contraceptive techniques available to limit fertility, the most effective apart from abstinence or sterilisation, and most widely used, is the contraceptive pill (often called simply 'the pill'). First produced in 1951, and used but modified since that date, it works by interfering with the natural menstrual cycle of the human female and preventing ovulation. If used correctly it is over 99% effective in preventing pregnancy.



The most frequently used type of pill is known as the **combined contraceptive pill** which contains low doses of both of the steroid hormones oestrogen and progesterone in synthetic form. The effect of the pill is to prevent release of both LH and FSH via the negative feedback inhibition by oestrogen and progesterone. Without these hormones follicles do not develop in the ovary each month and are thus an ovum is not released. To achieve this effect the pill must be taken daily for 21 days in each 28 day cycle. In the 7 pill-free days which follow, the uterus lining breaks down in response to the withdrawal of progesterone, giving a type of light menstrual period. (In biological terms this is not strictly necessary but is built into the design of the contraceptive pill so that a semblance of normal cycles is achieved).

The contraceptive pill also exerts effects on other parts of the reproductive tract. Firstly it causes the mucus in the cervix region to thicken, making it more difficult for sperm to enter, and secondly it limits the thickness of the endometrium, making it more difficult for implantation of a fertilised ovum. If a progesterone only pill, the 'mini pill' is taken the effects are limited to these changes, inhibition of ovulation does not necessarily occur.

The development of other types of contraception based on hormones is continuing. One method which is already in use involves implanting tubes containing progesterone crystals under the skin which will release a constant low level of the hormone sufficient to reduce fertility. These may last for up to 5 years and have the advantage over the pill in that they do not need to be taken daily.

Treating infertility

Infertility, the inability to achieve pregnancy, easily or at all, is a growing problem in the developed world. Up to 1 in 8 couples in the UK may be affected. The causes are varied and can be associated with either the male partner, the female partner, or both. Causes include the long term consequences of some sexually transmitted diseases, genetic factors, physiological factors (including hormonal imbalance), psychological factors, environmental factors (exposure to some pollutants or to radiation), and increasing age of women trying to conceive. Whatever its cause infertility is a serious and distressing problem for many couples and it should be noted that whilst medical technology is improving the options for such couples, many of the treatments are lengthy, expensive and have low success rates. There are also a range of ethical and moral issues associated with some forms of infertility treatment which are discussed later.

In about 20 % of women who fail to conceive the underlying causes are hormonal. Hormonal imbalances can prevent the development of follicles or prevent the release of ova. In both cases treatment using synthetic or naturally occurring female hormones is highly successful in restoring fertility. A range of treatments is available most being based around stimulating the release of either FSH, LH or both. The first approach is to use a cheap synthetic anti-oestrogen drug called Clomiphene to stimulate the production of FSH and LH. If this is unsuccessful, FSH and LH themselves, may also be taken to stimulate the cycle at appropriate points. This treatment is more expensive since these hormones must be purified from the urine of post-menopausal women or produced by recombinant micro-organisms.

Where other infertility problems exist (including blocked oviducts in a woman and low sperm count of the male partner) treatment relies on various types of in vitro fertilisation (IVF). IVF involves the extracting of both ova and sperm from the body, encouraging fertilisation outside the body, and then transferring any resultant zygotes back into the uterus of a woman (who may or may not be the donor of the ova). In order to extract large numbers of ova to allow for several attempts at IVF, both FSH and LH are commonly used to encourage multiple ovulation.

A further use of female hormones is in hormone replacement therapy (HRT) used to treat some of the unpleasant symptoms of the menopause in older women. In such women, synthetic oestrogens are given to make up for the decline in natural production which occurs as the menstrual cycle stops. This has many benefits: it reduces some of the physiological and psychological changes that often occur at the menopause.

◆ CHECKPOINT SUMMARY

- ◆ Contraception can be defined as the prevention of conception (fusion of male and female gametes). The most widely used is the contraceptive pill
- ◆ The 'pill' works by interfering with the natural menstrual cycle of the human female and preventing ovulation
- ◆ The commonest used type of pill is the combined contraceptive pill which contains low doses of hormones oestrogen and progesterone in synthetic form
- ◆ The effect of the pill is to prevent release of both LH and FSH via the negative feedback inhibition by oestrogen and progesterone
- ◆ To achieve this effect the pill must be taken daily for 21 days in each 28 day cycle. In the 7 pill-free days which follow the uterus lining breaks down in response to the withdrawal of progesterone, giving a type of light menstrual period
- ◆ The contraceptive pill also exerts effects on other parts of the reproductive tract. Firstly it causes the mucus in the cervix region to thicken, making it more difficult for sperm to enter; and secondly it limits the thickness of the endometrium, making it more difficult for implantation of a fertilised ovum
- ◆ If a progesterone only pill, the 'mini pill' is taken the effects are limited to these changes: inhibition of ovulation does not necessarily occur
- ◆ The development of other types of contraception based on use of hormones are being investigated. One method which is already in use involves implanting tubes containing progesterone crystals under the skin which will release a constant low level of the hormone sufficient to reduce fertility
- ◆ In about 20 % of women who fail to conceive the underlying causes are hormonal. Treatment using synthetic or naturally occurring female hormones is highly successful in restoring fertility
- ◆ A range of treatments for infertility in the female are available based around stimulating the release of either FSH, LH or both
- ◆ Both FSH and LH are commonly used to encourage multiple ovulation for in vitro ('test tube') fertilisation.

HORMONES in DOMESTIC ANIMALS

Superovulation and embryo transfer

As discussed above, artificial insemination gives farmers the ability to select semen from male animals showing particular desirable characteristics, so improving the quality of his stock. Recently technology has become available to allow selection of breeding females in a similar, although slightly more limited way. This procedure involves manipulating the reproductive biology of females (usually cows) by superovulation and embryo transfer and involves the following stages:

- ▼ Cattle, or other farm animals are treated with hormone injections to stimulate the development and release of much larger numbers of ova than would naturally occur. As in humans, the main strategy involves using FSH and LH at appropriate times. Theoretically up to 50 ova may be released per cycle, but generally it is between 3 and 8.
- ▼ When ovulation occurs (detected by the signs of oestrus mentioned above, or by calculation of time since LH injection) the cow is artificially inseminated.
- ▼ The fertilised ova are allowed to develop for up to 7 days and are then washed out of the uterus before implantation occurs.
- ▼ The embryos are frozen and stored until required.
- ▼ Embryos are then introduced into the uterus of cows other than those from which they were obtained; they implant and develop until full term. This process is known as **embryo transfer**. It should be noted that whilst embryos may be implanted in females from the same farm they may also be sold for implantation in cows in different regions or countries.

By this process a single high quality cow may produce a very large number of offspring allowing the quality of a herd to improve very quickly. Estimates from the 1980s show that superovulation of a cow is possible every 2-3 months. Each time 6-7 normal embryos will result from artificial insemination, of which 3-4 will successfully develop into calves. This gives a total of 15-18 calves per donor female per year. As these calves grow up their qualities in terms of meat and milk production can be assessed allowing farmers to rapidly ascertain the best cattle to breed from.

There are variants on the type of embryo transfer described above and the reasons for performing it. One main aim is to use embryo transfer to establish twin pregnancies in cattle where twinning is uncommon. This can potentially double productivity. Twin pregnancies are most often obtained by transferring two embryos into a cow who has not been inseminated, although they may be added to an existing zygote in the uterus of a cow who has already been inseminated. In some cases the sex of embryos may be determined before transfer. This is valuable to farmers as it ensures that they can achieve desired ratios of male to female cattle (more males than females required for beef farming and vice versa for dairy). The embryos may also be split to produce clones (genetically identical individuals) before transfer.

Synchronisation of oestrus

The synchronisation of oestrus cycles in flocks of sheep (ewes) or herds of cows is a very desirable way of manipulating animal reproduction. The aim of this process is to ensure that all animals in the herd come into oestrus on approximately the same day and can be mated or artificially inseminated at the same time. This allows substantial savings for the farmer in terms of both time and money, as well as improving conception rates and ensuring that the entire flock will be producing offspring at the same time the following season. Synchronised oestrus is particularly desirable in ewes, as opposed to cows or sows for two reasons: firstly these animals have distinct breeding seasons and cannot become pregnant outside of this season and secondly, many flocks of sheep live in isolated hill regions. It is much more efficient to herd the sheep together to start treatment and then again for mating or A.I.

The process of synchronisation is based on the administration and then withdrawal of the female hormone progesterone. Progesterone inhibits FSH and LH secretion and hence prevents the development of follicles and the release of ova. The process is as follows:

- ▼ All ewes in a flock are treated with progesterone for 5-9 days. The hormone is administered in one of three ways: orally, via an implant under the skin, or via a sponge in the vagina.
- ▼ The progesterone is stopped in all ewes on the same day. This removes the inhibition of FSH and LH and allows follicles to start development in the ovaries of the ewes.
- ▼ Several days later the ewes come into oestrus.
- ▼ A ram is obtained and used to fertilise all the sheep at the same time. Alternatively A.I. may be used. This may ensure higher fertilisation rates, be quicker, prevent the spread of disease and allow semen to be chosen from a wide range of rams.
- ▼ All ewes who were fertilised produce lambs at approximately the same time.

In addition to its use in synchronising oestrus during the breeding season, the use of hormonal treatments has also allowed flocks of ewes to come into oestrus (synchronised) at other times of the year. In these cases progesterone is given as described above but an extra hormone, gonadotrophin, is required to stimulate FSH and LH secretion. Light stimulation is sometimes used as well since the breeding season is triggered by changes in light intensity. Such manipulation may be used to shift the timing of oestrus and hence of lambing to more suitable times of the year based on known or predicted environmental or economic conditions. In some cases, where environmental conditions permit, farmers may divide their flocks into two, having half mating in October and lambing in March and the rest mating in July and lambing in December.

Milk production in cows

Over the thousands of years of animal husbandry, cows have been selectively bred to give high milk yields. Traditional breeding techniques combined with the modern use of A.I. and embryo transfer and improved understanding of bovine (cow) nutrition and management has led to enormous increases in yield in domestic breeds compared to wild cattle. Even though a high yielding cow may produce over 40 litres of

milk per day (up to 12,000 litres/year) there is still an interest in further boosting production. One reason for this is that in many countries (including Britain), prices paid to farmers for milk are very low, forcing farmers to maximise productivity if they are to make a profit. One solution to this is to boost production using hormones.

The hormone BST, (bovine somatotrophin), is a pituitary growth hormone which occurs naturally in young cattle. Its function is to increase cell division, protein synthesis and growth. Trials with BST, which is now produced by genetically engineered bacteria, showed that injecting dairy cows with the hormone led to a 10-15% increase in milk yield, presumably through an increase in growth and activity of milk producing tissue in the udder. Some of the advantage was offset by the fact that cows injected with BST require more food, but a net profit still resulted. These early trials showed no apparent change in behaviour, health or reproductive capacity of the cows.

Although BST is approved for use in cows in the USA, the hormone is banned in the European Union. This decision followed public protest as well as further scientific investigations of cows treated with BST which did suggest some health problems, including reduced resistance to disease.

Moral and Ethical Issues associated with interventions in reproduction

Any intervention in the process of reproduction is likely to be considered unethical by some groups in society. One widely held view is that increased availability of contraceptives, and particularly of the contraceptive pill, encourages sexual promiscuity. In addition, some religions, including Roman Catholicism and Islam condemn the use of contraceptives of any kind, arguing that limiting procreation is against God's will. Such religions do indeed value large families, and in many parts of the world the large family does provide an effective support system for elderly people. In opposition, however, many other groups would point to the benefits to both individuals and societies that contraceptive technology has brought. In dramatically decreasing birth rates it has improved the physical and emotional well-being of women across the world. In limiting family size it has also helped to contribute towards improvements in child health and economic status in many regions.

Intervention in human reproduction to treat infertility (mainly that involving IVF) is also the subject of much debate. Although the number of people undergoing such treatments is very small compared to the numbers using contraception, the issues raised concern society as a whole. Increasingly issues concerning the rights of individuals to have children are becoming high profile legal cases, emphasising the fact that medical and scientific advances in this field must be matched by progress in the fields of ethics and law.

A major argument exists over the fate of spare embryos (or pre-embryos) that have been conceived by IVF. There is a case for using them in medical research; particularly research associated with improving understanding of infertility and the development of new techniques to treat it. Others, however, believe that embryos are new individuals and that this would be immoral or unethical. As with discussions surrounding abortion one of the issues here concerns the right to life and the ownership of the embryos.

Applications of Gene Technology

Contents

Principles of genetic engineering

- ▼ The transfer of genes from one organism into another
- ▼ The use of restriction endonuclease enzymes to extract the relevant section of DNA
- ▼ The use of ligase enzyme to join this DNA into the DNA of another organism.

The polymerase chain reaction

- ▼ The process of artificial and repeated DNA replication in the laboratory by the polymerase chain reaction (PCR)
- ▼ The use of PCR, radioactive labelling and electrophoresis to determine the sequence of nucleotides in DNA.

Genetically engineered microorganisms

- ▼ Microorganisms as recipient cells during gene transfer
- ▼ Plasmids as vectors to incorporate selected genes into bacterial cells
- ▼ Use of microorganisms to clone a transferred gene.

Genetic markers

- ▼ Genetic markers in plasmids, such as genes which confer antibiotic resistance, and replica plating as techniques to detect the bacterial cells that contain genetically engineered plasmids.

Large scale culturing

- ▼ The culture of bacteria containing the transferred gene on a large scale in industrial fermenters
- ▼ Useful substances produced by using genetically engineered microorganisms, including antibiotics, hormones and enzymes.

Gene therapy and cystic fibrosis

- ▼ The cloning of healthy genes & their use in replacing defective genes
- ▼ The transmembrane regulator protein, CFTR mutation & its effects
- ▼ The symptoms of cystic fibrosis related to the malfunctioning of CFTR
- ▼ Techniques that might possibly be used to introduce healthy CFTR genes into lung epithelial cells including:
 - use of a harmless virus into which the CFTR gene has been inserted
 - wrapping the gene in lipid molecules that can pass through the membranes of lung cells.

Genetically modified animals

- ▼ Genetically engineered sheep to produce alpha-1-antitrypsin and the treatment of emphysema and cystic fibrosis.

Evaluation of genetic engineering

- ▼ The ethical, social and economic issues involved in the use of genetic engineering in medicine and in food production.

Introduction

In 1952 Hershey and Chase proved that DNA was the genetic material. The following year the double helix structure of DNA was elucidated, followed by the unravelling of the genetic code and the cloning of the first genes in the 1960s. From this point onwards the stage was set for direct human manipulation of DNA and the rise of DNA technology over the past four decades. Now, early in the twenty first century, the pharmaceutical and food industries are already reaping the benefits of genetically engineered organisms; trials are underway for gene therapy which might one day help to cure sufferers of severe genetic diseases; forensic science is benefiting from DNA fingerprinting; and evolutionary biologists are experimenting with the cloning of ancient DNA. In this rapidly developing branch of science there are many potential benefits to individuals and to society as a whole, and possibly many disadvantages.

PRINCIPLES OF GENETIC ENGINEERING

Genetic engineering is the manipulation of the DNA of an organism. It primarily involves removing genes from one organism (the donor) and inserting them into another organism (the recipient or host). The recipient organism is known as a **transgenic** organism. The donor organism may be of a different species, phylum, or even kingdom from that of the recipient organism. This is made possible by the fact that DNA has a universal code in all living organisms: genes are simply sequences of the four bases A, C, T and G whether they come from a human, a bacterium or a buttercup.

Genetic engineering achieved its first results in the 1970s using bacteria as the recipient organisms. Since that time a wide range of transgenic bacteria and fungi have been produced. More recently transgenic plants with features such as herbicide resistance, have begun to be grown on a large scale, and transgenic animals are being investigated for the production of medical products.

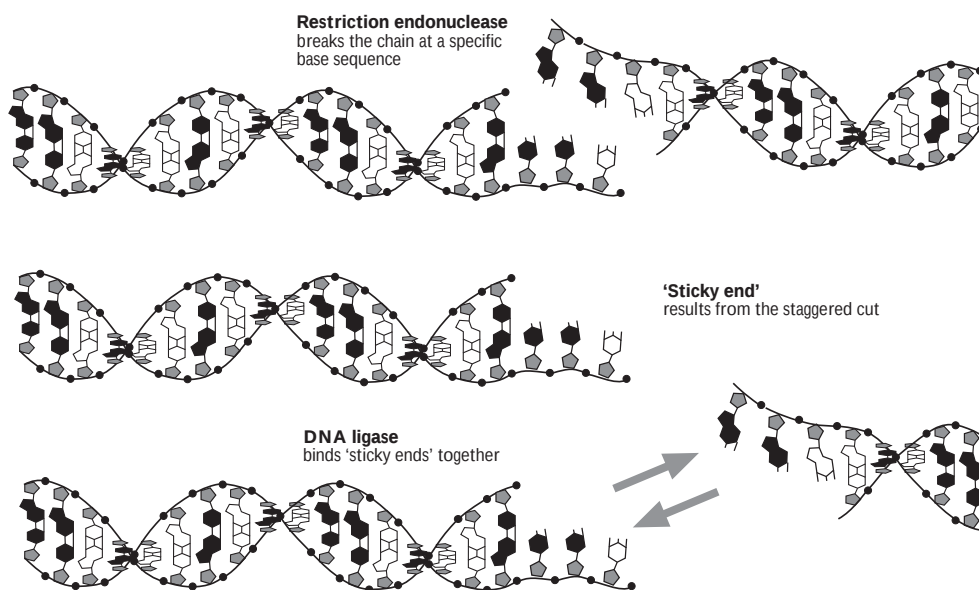
Genetic engineering involves several complex stages which differ depending on the organisms involved. All are highly specialised and technically difficult, but all centre around three basic stages:

- ▼ The desired gene must be found in the DNA of the donor organism or created from the mRNA of a known protein.
- ▼ The desired gene must be removed from the DNA of the donor using **restriction enzymes** (enzymes which cut DNA at specific points and allow removal of a small section: see below).
- ▼ The desired gene must be inserted into the DNA of the recipient organism. Having cut the DNA of the recipient organism using restriction enzymes, **ligase enzymes** are used to join the new gene permanently into its own DNA.

Restriction enzymes or restriction endonucleases are enzymes derived from bacteria which cut DNA at specific base sequences. Such enzymes will 'recognise' particular DNA sequences known as recognition sites, attach to these, and cut the DNA at specific restriction sites. There are several thousand types of restriction enzymes now in use whose names reflect the bacterial species from which they were extracted; thus EcoR1 is derived from *E. coli* and HindI from *Haemophilis influenzae*. In nature these enzymes allow bacteria to cut out sections of unwanted DNA which have been inserted by viruses.

Each restriction enzyme is specific to one base sequence and will digest or 'cleave' the nucleic acid (DNA) whenever such a site is encountered but at no other point. Some cut straight through both strands of DNA creating 'blunt ends', whilst others produce a staggered cut or 'sticky end' which is useful in genetic engineering. In genetic engineering restriction enzymes must be chosen which cut the donor DNA either side of the desired gene. The same enzyme will then be used to cut the DNA of the recipient organism or vector to allow the gene to be inserted.

Action of restriction and ligase enzymes



POLYMERASE CHAIN REACTION (PCR)

PCR is a technique used to amplify (make many copies of) a DNA molecule or part of a DNA molecule. Based on an understanding of the semi-conservative mode of DNA replication in nature, PCR has revolutionised genetic technology. It theoretically allows any number of copies of DNA to be produced from a single DNA molecule (the template). Often it is used to amplify a known section of DNA only. PCR is crucial in generating copies of DNA for use in such processes as: gene sequencing, genetic fingerprinting and genetic screening for diseases.

Amplifying a piece of DNA by PCR requires the 4 'ingredients' listed below.

- ▼ DNA. Theoretically a single DNA molecule is sufficient, but usually a larger amount of DNA is used.
- ▼ Primers: These are short single stranded pieces of DNA which bind to the parent DNA strands and initiate the synthesis of a complete new strand of DNA on the template of the parent strand. One primer is needed for each parent DNA strand.
- ▼ A supply of each of the 4 nucleotides containing the bases A, C, T and G. The bases on the free nucleotides pair with their complementary bases on the DNA parent strand (A with T and C with G) thus synthesising the new strand.
- ▼ The enzyme Taq polymerase. This enzyme works like human DNA polymerase, catalysing the attachment of the complementary bases to one another. Taq polymerase is, however, thermostable (temperature resistant) as it is derived from the bacterium, *Thermus aquaticus*, which inhabits hot springs. This is important as PCR requires high temperatures to work.

The reaction mixture made of these 4 ingredients is placed in an individual well on a plastic plate, or in individual tubes with a layer of oil on top to prevent evaporation. The reactions involve alternate heating and cooling of this mixture, and are performed by a PCR machine. The machine can be programmed for different numbers of cycle: the more cycles the more copies of the DNA are produced.

Sequence of events

For simplicity the stages of PCR are described here as if a single complete

DNA molecule is used as the starting point.

- a) PCR mixture is heated causing the DNA double helix to unwind exposing the bases.
- b) The mixture is cooled and primers bind to the ends of each DNA strand. This is known as annealing.
- c) Taq polymerase allows free nucleotides to pair with the exposed bases on both DNA strands. A pairs with T and C with G. Binding begins immediately after the primer sequence and continues until the whole complementary strand has been synthesised. Two new DNA double helices thus result. Each contains one of the original parent strands. One complete cycle has now been completed.
- d) The process is repeated starting with two copies of the DNA and ending with four. (Each DNA molecule unwinds, has a complementary strand synthesised and winds back up). As the

process is repeated over and over again the number of copies of the DNA increases geometrically: $2 \rightarrow 4 \rightarrow 8 \rightarrow 16 \rightarrow 32 \rightarrow 64 \rightarrow 128$ etc. Several million copies may be made in the space of a few hours.

- e) The DNA produced is used in genetic fingerprinting, genetic screening or other genetic studies. Alternatively it may be frozen and stored for future analysis.

GENETICALLY ENGINEERED MICROORGANISMS

In biology the term microorganism is used to describe any single celled organism which can only be seen using a microscope. It therefore includes bacteria, unicellular fungi and some members of the protist kingdom. Many different types of bacteria and more recently some types of yeast (a single celled fungus) have been used in genetic engineering.

Bacteria are ideally suited to genetic engineering. One of the reasons for this is that they contain small circles of DNA known as plasmids which act as vectors by which new genes can be introduced into bacterial cells. The first stage of genetic engineering is to identify and isolate the desired gene from the donor organism. This is very difficult and involves a range of techniques beyond the scope of this text. Instead we will consider the process the stages from this point onwards, assuming that the desired gene is ready to be inserted:

Stage 1

Inserting genes into the plasmid vector

- The plasmids must be removed from bacterial cells. Bacteria cells are broken open using high temperatures or chemical means. The contents are centrifuged and plasmids purified.
- The plasmid DNA is cut open using a restriction enzyme. The same restriction enzyme is used as was used to cut the donor gene from donor DNA. In both cases 'sticky ends' are produced which helps the insertion of the gene.
- The donor genes are mixed with the plasmids.
- Some plasmids take up donor DNA. Attraction occurs between sticky ends but insertion of the gene is aided by ligase enzymes. The DNA of the plasmid is now called recombinant DNA.

Stage 2

Introducing vector DNA into the host cell (transformation)

- Plasmid vectors containing recombinant DNA added to a culture of bacteria (often *E.coli* bacteria are used at this stage although the plasmids may come from other species of bacteria.)
- Calcium ions and heat are used to make the cell membrane of the bacterium permeable to plasmids.
- Plasmids successfully enter some bacterial cells.

Stage 3

Cloning DNA (producing many copies of the recombinant DNA)

Bacteria from Stage 2 are then grown using suitable medium. Since bacteria reproduce at a very fast rate billions of cells can be created

◆ CHECKPOINT SUMMARY

- ◆ Genetic engineering is the manipulation of the DNA of an organism. It primarily involves removing genes from one organism (the donor) and inserting them into another organism (the recipient or host). The recipient organism is known as a transgenic organism.
- ◆ There are three basic stages:
 - ◆ The desired gene must be located in the DNA of the donor organism or created from the mRNA of a known protein.
 - ◆ The desired gene must be removed from the DNA of the donor using restriction enzymes which cut DNA at specific points and allow removal of a small section.
 - ◆ The desired gene must be inserted into the DNA of the recipient organism. Having cut the DNA of the recipient organism using restriction enzymes, ligase enzymes are used to join the new gene permanently into its own DNA.
- ◆ The Polymerase Chain Reaction (PCR) is a technique used to amplify (make many copies of) a DNA molecule or part of a DNA molecule.
- ◆ PCR has revolutionised genetic technology. It theoretically allows any number of copies of DNA to be produced from a single DNA molecule (the template).
- ◆ PCR, radioactive labelling and electrophoresis in which fragments of different size are separated and identified allows the determination of the sequence of nucleotides in DNA.

in a short space of time. Every time the bacteria containing recombinant plasmids divide copies of the plasmid containing the desired gene are passed on. A single bacterium may have many copies of the plasmid, thus increasing the number of cloned genes present.

Stage 4

Selecting the transformed bacteria using GENETIC MARKERS

Stages 1 and 2 described above are far from perfect. In stage 1 many plasmids do not take up the donor DNA, and in stage 2 many bacteria do not take up plasmids. Of those bacteria that do take up plasmids, only those taking up plasmids containing the desired gene will be transformed. To check which bacteria from the clones grown in stage 3 have been transformed (and are hence useful for producing a product) a special technique involving marker genes for antibiotic resistance is often used.

Before being used as vectors, plasmids are produced which have two genes for antibiotic resistance. One gene will make the bacteria containing the plasmid resistant to the antibiotic ampicillin, and the other will confer resistance to the antibiotic tetracycline. When a plasmid takes up a gene from the donor organism, as described in stage 1, the gene is inserted in the middle of the tetracycline resistance gene sequence owing to the presence of restriction enzyme sites. This inactivates the tetracycline resistance gene. The ampicillin gene is unaffected.

Bacteria transformed by recombinant plasmids should therefore be resistant to ampicillin but will not grow on agar containing tetracycline. Bacteria which have taken up normal plasmids which do not contain recombinant DNA should be resistant to both antibiotics and will grow on agar containing both antibiotics.

Replica plating

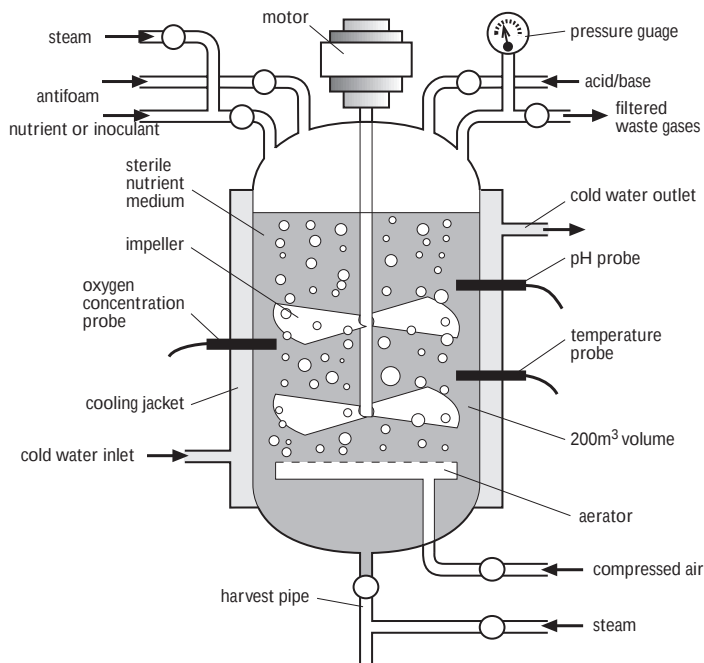
To determine which bacteria have been transformed the following steps are taken:

1. Bacteria from stage 3 above are placed onto an agar plate containing ampicillin.
2. When colonies of bacteria have developed, a sterile pad is placed on top of the colonies. This allows some bacteria from each colony to be picked up.
3. The pad containing the bacteria is pressed onto a second agar plate containing ampicillin and tetracycline. The use of the pad reproduces the same pattern of colonies on the new plate.
4. Colonies of transformed bacteria fail to grow and are destroyed by the antibiotic. Bacteria without recombinant plasmids grow.
5. Colonies of transformed bacteria on the original plate can now be identified. These are then cultured. The remaining colonies are discarded.
6. Once transformed bacteria have been selected they can then be cultured to produce the desired gene product. Techniques to maximise bacterial growth and hence production have developed alongside genetic engineering. A range of valuable enzymes, hormones and other proteins are now produced rapidly, cheaply and safely by microorganisms in fermenters.

LARGE SCALE CULTURING OF GENETICALLY MODIFIED MICROORGANISMS

Growth of bacteria in industrial fermenters (large specialised tanks) involves the same basic principles as the small-scale growth of microorganisms in a laboratory. Bacteria will only grow at their optimum rate if provided with the following basic conditions:

1. A suitable nutrient medium in which to grow. This should contain simple organic sources of carbon and nitrogen (such as simple sugars and amino acids) and a range of vitamins and minerals dissolved in water.
2. Suitable environmental conditions, including an optimum pH and temperature for enzyme activity. Oxygen may or may not be required. Toxic waste products which inhibit growth should be removed.
3. Complete absence of competition or predation from other microorganisms. To achieve this the fermenter is sterilised before use and all substances added are also sterile.



Note in particular the inlet pipes by which nutrients are added, the temperature and pH monitors, the measures to exclude other microorganisms, the outlet pipes by which the product is harvested and the paddle wheel to keep the contents well mixed. In initial stages of culturing genetically engineered microorganisms small fermenters with volumes of 2-200 litres are used. Once established, however, vessels up to 200,000 litres may be used. With fermenters of this size, several hundreds of tonnes of product can be generated per day.

There are two main types of fermentation: **batch** fermentation and **continuous** culture. Batch fermentation is where a single culture of microorganisms and a single batch of nutrient medium is used to produce one batch of product. The microorganisms are then discarded and the process begins again. In continuous culture a colony of microorganisms is maintained over a long period of time. Small quantities of nutrient medium are added continuously and the product is removed regularly.

A range of products are now produced in fermenters by genetically engineered microorganisms. Some of these are shown below.

Product/function	Microorganism involved	Uses
Insulin	Bacteria and yeast	To treat diabetes
Growth Hormone	Bacteria	Promote growth in children with deficiencies of pituitary gland
Bovine somatotrophin (BST)	Bacteria	Increase milk production in cattle
Penicillin (antibiotic)	Fungus	To treat bacterial infections
Hepatitis B antigens	Bacteria	Safe vaccine against hepatitis B.
Chymosin (an enzyme like rennin)	Bacteria	Cheese making (vegetarian cheese)

◆ CHECKPOINT SUMMARY

- ◆ Genetically engineered microorganisms include many different types of bacteria and more recently some types of yeast
- ◆ Plasmids are often used as vectors to incorporate selected genes into bacterial cells.
- ◆ To check which bacteria from the clones have been transformed (and are hence useful for producing a product) a special technique involving marker genes for antibiotic resistance is often used.
- ◆ Bacteria transformed by recombinant plasmids being resistant to ampicillin but susceptible to tetracycline.
- ◆ Large scale culturing of genetically modified microorganisms involves techniques to maximise bacterial growth and hence production
- ◆ Once established vessels up to 200 000 litres may be used. With fermenters of this size, several hundred tonnes of product can be generated per day.
- ◆ There are two main types of fermentation: batch fermentation and continuous culture. Batch fermentation is where a single culture of microorganisms and a single batch of nutrient medium is used to produce one batch of product.

GENE THERAPY AND CYSTIC FIBROSIS

The replacement or manipulation of defective genes in the cells of individuals suffering from a variety of diseases represents a major goal in the application of genetic engineering technology to human health. Termed 'gene therapy' this branch of medicine is still at the research stage, although it is anticipated that it could revolutionise the treatment of genetic disease in the future by effectively curing the disease 'at source'. Initial work has been done with single gene disorders such as cystic fibrosis, but it has been suggested that gene therapy could extend to the treatment of some cancers and even HIV/AIDS.

There are two main approaches to gene therapy. The first, and the only one currently being investigated in humans is known as '**somatic gene therapy**'. This approach involves inserting 'normal' genes into the DNA of some of the body cells of an affected individual. Target cells might, for example, be those of the lung as described below. Whilst this could cure an individual of a genetic disease it would not affect the gametes produced and hence the inserted genes would not be passed on to future generations. In contrast, germ line gene therapy involves the introduction of genes into sex cells or fertilised egg cells. In this case the new gene would be found in all somatic cells and all germ cells and would thus change the genetic makeup of future individuals. The ethical implications of such germ line gene therapy are considerably greater than with the somatic form. For this reason research into germ line gene therapy in humans is currently banned.

The procedure of gene therapy involves three main stages, once the cause of a genetic disease has been established and the gene or genes responsible located.

- ▼ Firstly the normal gene must be isolated, removed from a human cell and cloned using bacterial cloning methods already described.
- ▼ Secondly the normal gene(s) must be inserted into a suitable vector. Possible choices of vectors are: viruses, liposomes and naked DNA.
- ▼ Thirdly the genetic material must be transferred into the patient. In some cases, as with defects of the blood cells this may be done outside the body using cells extracted from the patient which can then be modified and returned. In other cases the DNA must be delivered into the body of the patient via aerosols or other methods.

Cystic fibrosis

Cystic fibrosis is one of the most common non-sex chromosome (autosomal) recessive genetic diseases and affects about 1/2000 babies born in the UK. About 1:23 of the population are carriers of the cystic fibrosis allele which is found on chromosome 7. The disease which primarily affects the lungs, pancreas and liver is characterised by the production of thick, sticky mucus by epithelial cells in these regions. As well as causing severe breathing difficulties the mucus also leads to numerous bacterial infections. In spite of antibiotics and other drug treatments and regular physiotherapy to help move the mucus out of the lungs, CF usually causes death by early adulthood. CF is caused by a faulty protein known as CFTR (cystic fibrosis transmembrane regulator) which regulates transmembrane chloride ion channels in epithelial cells. A deletion of 3 bases in the DNA code of the CFTR gene means that one amino acid (phenylalanine) is missing from the CFTR protein which is produced. This change in the primary structure of the protein alters its secondary and tertiary structures, preventing it from responding to signals to open chloride channels in cells. The overall effect of this is a build up of ions inside the cells which prevents water leaving by osmosis as it would normally do. Thus mucus released by cells is thicker than in normal individuals and sticks to cells causing inflammation and tissue damage, encouraging infections and preventing normal functioning of the organs concerned.

Owing to the lack of effective treatments available to CF sufferers there has been much interest in gene therapy for this disease. The aim in this case is to replace the faulty CFTR gene in lung cells with the normal gene for CFTR production along with its control sequence, thus allowing production of the normal protein in cells of the lungs. Two delivery techniques have so far been tried to deliver cloned copies of the CFTR gene to human cells.

- ▼ delivery via an adenovirus vector. This is a DNA virus of the family which causes colds. The virus has its pathogenic (disease causing) genes removed and the DNA for CFTR inserted. When the virus invades cells the DNA for CFTR is released.

Advantages of this system are that the vector will readily infect non-dividing cells and has a natural attraction to cells of the lung region. Disadvantages are that the virus may be attacked by the immune system, and that the DNA is unstable.

▼ delivery via liposomes. These particles are created in the laboratory by the fusion of the CFTR DNA (negatively charged) with positively charged lipid particles. They are taken up by the cell surface membrane of lung cells and the CFTR DNA can become functional inside the cell.

The advantage of this method is that it doesn't cause an immune response, but the disadvantage is that it is a very inefficient means of delivering DNA since most of it gets destroyed by lysosomes.

Both delivery techniques were first tried out in vitro (in the laboratory) using extracted lung cells, and then in experimental animals (where the CFTR gene had been removed). More recently trials in small numbers of humans have been performed. In these the adenovirus vector was used to deliver the CFTR gene, via an aerosol spray, to cells in the nasal passages and lower respiratory tract. Liposome vectors have so far only been used to deliver the gene to the nasal passages, also via an aerosol spray.

Human trials have provided evidence of gene transfer taking place between vector and human cells in up to 50% of cases. In addition, there has been more limited evidence of production of functional CFTR protein correcting the defect. In all cases where functional improvement occurred this was restricted to a period of 7-10 days which can be explained by the fact that the cells targeted by gene therapy do not divide and consequently will die and be lost. Thus whilst some progress has been made into gene therapy for CF, cure of this disease is still a long way off.

GENETICALLY MODIFIED ANIMALS

Several human proteins of great medical value are now produced by genetically engineered micro-organisms. In some cases, however, microorganisms are not well adapted to this task often because they lack specific enzymes which are required to modify the proteins produced and allow them to become biologically active. For this reason, scientists have developed techniques of genetically engineering mammals (farm animals) to produce human proteins. Such animals are sometimes termed 'animal bioreactors'.

In contrast to work with microorganisms, insertion of human genes into mammalian egg cells is very difficult and costly, whilst the care of farm animals is expensive and their reproductive potential limited. For these reasons such techniques must allow continued production and easy extraction of the protein without killing or harming the animal. The ideal solution is to have the protein secreted in milk. To achieve this the human gene is attached to a gene control sequence from the recipient mammal which ensures that the gene will only be 'switched on' in the tissues of the mammary gland.

Production of alpha-1 antitrypsin

Alpha-1 antitrypsin is a protein which is produced in the human liver and transported in the plasma. Its main function is to inhibit the enzyme elastase which is produced by white blood cells. If this enzyme is not inhibited (owing to a hereditary deficiency of alpha-1 antitrypsin) a form of early onset emphysema can develop whereby alveoli are destroyed and lung function considerably reduced. These patients make up a small percentage of emphysema patients and may or may not be smokers.

In recent years sheep have been genetically engineered to produce the alpha-1 antitrypsin enzyme using the procedure outlined below. Alpha-1 antitrypsin enzyme now makes up half of the protein content of the milk of these transgenic sheep. The enzyme can be easily extracted. Whilst clinical trials of this enzyme in humans is still awaited (May 2000) it has the potential to improve the treatment of patients with emphysema and cystic fibrosis. Currently these patients rely on alpha-1 antitrypsin enzyme extracted from human blood.

Stages in the production of alpha-1 antitrypsin by transgenic sheep

- ▼ Locate gene for alpha-1 antitrypsin and its control sequence in DNA of human liver cells
- ▼ Cut gene from DNA using restriction endonuclease
- ▼ Copy gene using PCR techniques
- ▼ Separate gene and control sequence using restriction enzymes
- ▼ Locate B-lactoglobulin gene and its control sequence in DNA from mammary cells of sheep
- ▼ Cut gene from DNA using restriction endonuclease and clone gene using PCR techniques
- ▼ Separate gene and control sequence using restriction enzymes
- ▼ Attach control sequence DNA from B-lactoglobulin to alpha-1 antitrypsin gene
- ▼ Use microinjection to deliver copies of hybrid transgene to fertilised sheep ovum
- ▼ Test for production of alpha-1 antitrypsin in milk of adult sheep using protein electrophoresis.

◆ CHECKPOINT SUMMARY

- ◆ Somatic gene therapy' involves inserting 'normal' genes into the DNA of some of the body cells of an affected individual. Target cells are the lungs in cystic fibrosis.
- ◆ Firstly the normal gene must be isolated, removed from a human cell and cloned.
- ◆ Secondly the normal gene(s) in the form of cDNA must be inserted into a suitable vector. Possible choices of vectors are: retroviruses, adenoviruses, liposomes and naked DNA.
- ◆ Thirdly the genetic material must be transferred into the patient.
- ◆ Cystic fibrosis is caused by a faulty protein known as CFTR (cystic fibrosis transmembrane regulator) which regulates transmembrane chloride ion channels in epithelial cells. A deletion of 3 bases in the DNA code of the CFTR gene means that one amino acid (phenylalanine) is missing from the CFTR protein which is produced.
- ◆ The overall effect of this is a build up of ions inside the cells which prevents water leaving by osmosis as it would normally do. Thus mucus released by cells is thicker than in normal individuals and sticks to cells causing inflammation and tissue damage, encouraging infections and preventing normal functioning of the organs concerned.
- ◆ Techniques to introduce healthy CFTR genes into lung epithelial cells include, delivery via a DNA virus of the family which causes colds; and wrapping the CFTR gene in lipid molecules. They are taken up by the cell surface membrane of lung cells and the cDNA can become functional inside the cell.
- ◆ Animals, e.g. sheep have been genetically modified to produce the alpha-1 antitrypsin enzyme used in the treatment of patients with emphysema and cystic fibrosis.

EVALUATION OF GENETIC ENGINEERING

Ethical, social and economic issues

In considering genetic engineering in its social and economic context it is worth reflecting on the fact that some kind of genetic modification of plants and animals has been in operation for thousands of years. Whilst early farmers did not know about genes or, of course, genetic engineering, their techniques of selective breeding and hybridisation dramatically altered gene frequencies and gene combinations in domestic organisms. The resultant changes in appearance and characteristics helped to create our familiar high yielding cereals, large, sweet fruits, and cows with high milk yields. In view of this it could be said that genetic engineering is at the modern end of a continuum of genetic change brought about by humans.

Yet for a variety of reasons the current methods of genetically modifying (GM) organisms, particularly plants and animals, is opposed by many people. Some likely factors behind this are the speed with which genetic engineering can operate, the potential for transferring genes between very different organisms and the greater public awareness of the process owing to media attention.

Food production One of the main arguments against GM foods is the concern over possible health risks. It is argued that plants containing genes from other organisms including in some cases bacteria and viruses may be unsafe, although there is no strong scientific evidence to suggest that there is any risk from foods currently in use.

GM crop plants are at the centre of a debate about the possibility of cross-pollination of wild plants, so spreading genes into these organisms. If, for example, genes for herbicide resistance were transferred to weeds then weeds might spread rapidly, compete with crops and upset natural food chains. On the positive side, however, it is argued that GM crops may actually benefit wildlife. If crops need fewer chemical herbicides, pesticides and fertilisers it could reduce pollution and the unnecessary killing of harmless organisms. Although, one of the first widely planted GM crops is a Maize which is resistant to a certain brand of herbicide.

The possible hazard of genetically engineered micro-organisms, including viruses, escaping from laboratories also raises safety issues. One particular worry is that bacteria used for a range of genetic engineering purposes often carry genes for antibiotic resistance which are used as markers. Since bacteria can exchange genetic material, they could transmit these genes to disease causing organisms which may then be resistant to treatment, adding to the already growing problem of natural resistance.

A further issue raised by the current debates on genetically engineered foods is the idea of consumer choice. It is argued that individuals should be able to choose whether or not to buy food containing GM ingredients, yet labelling of products does not make this possible. In other ways, though, genetic engineering can increase consumer choice: products such as 'Quorn' (a high protein meat substitute produced by a fungus) and vegetarian cheese (produced using a form of rennin from genetically engineered bacteria) were not available before genetic engineering. In addition GM foods may be cheaper, and for many people cost is the most important factor to consider.

Genetic engineering has also had an impact on animal products mainly via the use of genetically engineered molecules such as